

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE****In Re Application of: Frank Grosveld****Confirmation No. 1498****Serial No.: 10/693,308****Art Unit: 1632****Filing Date: October 24, 2003****Examiner: Anoop K. Singh****For: IMMUNOGLOBULIN 2****Customer No.: 34132****DECLARATION OF RUDOLF GROSSCHEDL, Ph.D.**

1. I have been engaged by Erasmus University to provide my opinion on certain issues concerning the above-identified application. I am being compensated at a rate of \$ 360 per hour to do so.
2. My short form *curriculum vitae* ("cv") is attached as Exhibit A.
3. I received a Masters of Science in biology from the University of Freiburg, Germany, in 1978. I received a Ph.D. in molecular biology from the University of Zurich, Switzerland, in 1982. I served as a Postdoctoral Fellow in the laboratory of Dr. David Baltimore, the Whitehead Institute and Massachusetts Institute of Technology, Cambridge, Massachusetts.
4. I am currently the Director, Max-Planck Institute of Immunobiology, Department of Cellular and Molecular Immunology, Stübeweg 51, 79108 Freiburg, Germany.
5. As Director of the Max-Planck Institute of Immunobiology, my duties include supervising research, hiring and mentoring of junior group leaders
6. As is evident from my cv, I have been involved in research regarding the expression of immunoglobulin genes and transgenic animals since at least 1988, and have published extensively on the topics. More specifically, I have been involved in research concerning tissue-specific expression of immunoglobulin genes in transgenic mice. I am on the editorial boards of three peer-reviewed journals – *Molecular and Cellular Biology*, *Genes and Development*, and *Immunological Reviews*.

7. I have reviewed the above-identified application, the correspondence with the examiner and the recent response from the inventor Professor Grosveld. I am familiar with the involved technology, namely single heavy chain antibodies. Single heavy chain antibodies are antibodies composed of heavy chains only; no light chains are present.
8. All claims of the above-identified application are currently under rejection by the U.S. Patent & Trademark Office ("the Office"). I have read the Final Rejection dated February 12, 2007. I have also read the Interview Summary dated June 1, 2007; the Request for Reconsideration ("Request") filed June 25, 2007; and the Advisory Action Before the Filing of an Appeal Brief ("Advisory Action") dated July 9, 2007.
9. In the Final Rejection, the Office rejected the claims under the following bases:
  1. For lack of enablement under 35 USC § 112, first paragraph;
  2. For being incomplete as omitting essential steps under 35 USC § 112, second paragraph;
  3. As anticipated by Ledbetter et al (WO 99/42077) under USC § 102(b); and
  4. For obviousness-type double patenting over the claims of co-pending Application No. 10/692,918.

I have been asked to address basis 1 above. The Office's view is that undue experimentation would be required to practice the invention. I respectfully disagree. The first basis for rejection is further broken down into four issues. Based on my expertise, I will be addressing the third and fourth issues.

10. The third issue is that the claims cover the use of any promoter, with any regulatory elements, in any non-human transgenic mammal. The Office alleges that, at the time of invention (which I understand to be at least April 24, 2001, the earliest priority date claimed), only the mouse was recognized by the art of record as a routinely manipulated animal, and that the art of record recognized the unpredictability of making transgenic animals other than mice (see page 8 of the Final Rejection). I do not agree that the art of record shows this.
11. It is well established that large tracts of human genomic sequence can be transferred into the mouse or other mammalian genomes using YACs. Moreover, human genes and regulatory elements when placed in a mouse or another mammalian background respond to tissue specific and developmental signals as would the endogenous homologue. This is

particularly well documented for the human immunoglobulin heavy and light chain genes which respond in a mouse background to antigen challenge in a B-cell specific manner leading to the production of human immunoglobulins (see Jacobovits, A (1994) *Current Biology*, 4, 761-763 and references cited therein) (**copy attached**). Human heavy and light immunoglobulin gene loci have also been expressed as transgenes in cattle (Kuroiwa et al., *Nat Biotech.*, 2002; 20:889-894, "Cloned Transchromosomal calves producing Human Immunoglobulin" (**copy attached**). Thus the mouse provides a valid model to evaluate gene expression with the expectation that if a gene is functionally expressed in the mouse it will also be expressed as a transgene in other non-human mammals. Thus for expression of a camelised human immunoglobulin heavy chain only gene loci the same YAC cloning strategy reviewed by Jacobovits has been followed, excepting that the final loci used are devoid of CHI, and comprise selected V gene segments ( in Janssens et al Llama VHH-segments, and in the recent data disclosed by Professor Grosveld in his declaration that I understand is being submitted concurrently - human VH segments). All regulatory elements known to ensure high levels of expression in B-cells are present in the YAC construct used in the specification as described in the prior art. In particular, the enhancer element required for the initiation of the recombination and expression of the IgH locus is located in the intron between the JH and C $\mu$  regions (Gillies et al., 1983, Grosschedl and Baltimore, 1985; Sen and Baltimore 1986, and references cited therein; **copies attached**; *see also* Serwe and Sablitzky (1993), cited on page 23, lines 10-11 of the specification), while the enhancers (LCR) that ensure high levels of the rearranged IgH transcripts regardless of position are located to the 3' side of the last constant region (mouse locus, Pettersson et al., 1990; human locus, Mills et al 1997; **copies attached**). Use of both the intronic enhancer and the 3'LCR was known to confer correct spatial and position-independent expression of a linked transgene in mice (Grosschedl et al., 1984; Lieberson et al., 1995; Chauveau et al., 1999; Janssens et al, 2006; **copies attached**). Both sets of elements were present on applicant's llama-human IgH locus. Briefly, applicant took PAC clone 1065 N8 (BABPAC Resource Center Oakland CA) containing the intronic enhancer sequences and Bac clone 11771 (Incyte Genomics, PaloAlto CA) containing the LCR. The intronic enhancer region was cloned into the final construct as a 120kb Sall fragment (i.e. similar

to Bruggemann et al., Figure 1 top line). The LCR was cloned as a combination of three fragments, BclI/BamHI (3.7kb containing HS1), BamHI (8kb containing HS1 and 2) and BamHI/BglII (7.5 kb containing HS4) into BlueScript and transferred to the final construct as a NotI/SalI fragment. The LCR sequences are the same as shown by Mills et al (page 846 Figure 1).

12. The presence of the Ig LCR is preferable but not essential. As noted above, its presence ensures that every transgene is transcriptionally active irrespective of its site of insertion in the host genome, an important consideration when working with larger mammals, such as cattle and sheep, with long breeding cycles relative to the mouse (Grosschedl et al., 1984; Lieberson et al., 1995; Chauveau et al., 1999), but not necessary for smaller mammals.
13. Since the regulatory elements present in an Ig heavy chain transgene comprising CH1 (in this instance human) determine B-cell specific expression in mice and cattle, then the introduction of a heavy chain only immunoglobulin loci (devoid of CH1) comprising the same regulatory elements into a non-human mammalian host should also result in the expression of the introduced transgene, the observed result in mice. Consequently if a human IgH locus devoid of CH1 was introduced into cattle, or for that matter any non-human mammal, it would be reasonable to expect based on the evidence available that the IgH gene (devoid of CH1) would be expressed and human heavy chain only antibody would be present in the serum. This conclusion is supported by Buelow and others who have published on the expression of human and humanised immunoglobulin genes in non-human mammalian species as diverse as rabbit, pig, sheep, and cow (see WO 02/12437 A2 and references therein, **copy attached**). Thus the elimination of CH1 domains from these immunoglobulin genes would predictably result in the production and circulation of heavy chain only antibody as observed by Janssens et al in transgenic mice.
14. Experimental conditions for the introduction of genes into the germ-line (transgenesis) have been optimised for rodents and other laboratory animals, also many animals of agricultural importance e.g. rabbit, pig, goat, sheep, cattle. Hence, the application of this technology as described and applied to mice could be followed by someone of ordinary skill in the art of the derivation of heavy chain only antibody in non-human transgenic

animals, following the guidance provided in the specification.

15. There is no scientific evidence to suggest that natural IgH regulatory elements active in one non-human mammal will not be active in another. In fact, the IgH regulatory elements have been well characterized, and I am not aware of reports in which a gene that is expressed faithfully in one mammal is not expressed in another mammal. Rare cases, in which poor expression has been reported, do not reflect the field and are typically due to the use of incomplete constructs.
16. The final issue of the first basis for rejection focuses upon the insertion of the vector into the mammals. Although acknowledging that insertion of the vector can be accomplished by either microinjection (into fertilized eggs) or through use of embryonic stem (ES) cells, the Office states that the method requires the use of ES cells, which the Office then states is restricted to mice (page 10 of the Final Rejection). Janssens et al., however, reports that the vectors were injected into fertilized mouse eggs, not ES cells. Use of ES cells, thus, is **not** required. I would expect that any route of introduction of the vector into the germ-line of any mammal would result in heavy chain antibody production by B-cells.
17. Based on the overwhelming evidence available it is not unreasonable to conclude that the invention (methods for the in vivo derivation of heavy chain only antibodies in transgenic non human mammals in response to antigen challenge) using the natural mammalian IgH regulatory elements as set out could equally be applied by those skilled in the art for the derivation of heavy chain antibody (devoid of CH1) as a result of antigen challenge in any non-human mammalian background in addition to the mouse, for example rat, rabbit, pig, goat sheep, cow, monkey, etc. Indeed, the inventor used human regulatory elements in the mouse with success. Thus, the route described for the derivation of heavy chain only antibody as exemplified in the application and by Janssens et al using the mouse, can be equally applied to any non-human mammalian system. Specifically, I note the following.
  - The introduction of transgenes into the germ-line of non-human mammalian systems was well established at the time of filing.
  - Regulatory elements that ensure high levels of B-cell specific IgH were well characterised and were known to be present in the human IgH genomic sequence

described, moreover these were known to be functional when incorporated in transgenes in non-human mammals at the time of filing.

- At the time of filing, there was a wealth of evidence to demonstrate that enhancer and LCR elements derived from one mammalian species (including human) are functional in other mammalian species.

18. I hereby declare that all statements made herein are of my own knowledge true and statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

2/3/08

Date

Rudolf Grosschedl

Dr. Rudolf Grosschedl

# **CURRICULUM VITAE**

**RUDOLF GROSSCHEDL, Ph.D.**

**TITLE**                    Professor of Biochemistry  
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## **DATE AND PLACE OF BIRTH**

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**CITIZENSHIP**    Austrian, USA.

## **PERMANENT RESIDENCY**

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## **EDUCATION**

1971-1972	University of Salzburg, Austria
1972-1978	University of Freiburg, Germany M.S. in Biology, Thesis topic: Structural analysis of the replication region of lambdoid bacteriophages Thesis advisor: Dr. Gerd Hobom
1978-1982	University of Zurich, Switzerland Ph.D. in Molecular Biology Thesis topic: Functional analysis of an histone gene promoter Thesis advisor: Dr. Max L. Birnstiel

## RESEARCH EXPERIENCE

- 1977-1978 Undergraduate Student, Laboratory of Dr. Gerd Hobom  
University of Freiburg, Germany
- 1978-1982 Graduate Student, Laboratory of Dr. Max Birnstiel  
University of Zurich, Switzerland
- 1982-1985 Postdoctoral Fellow, Laboratory of Dr. David Baltimore  
Whitehead Institute and Massachusetts Institute of Technology  
Cambridge, Massachusetts, USA

## POSITIONS HELD

- 1982-1985 Postdoctoral Fellow  
Massachusetts Institute of Technology  
Cambridge, Massachusetts, USA  
Advisor: Dr. David Baltimore
- 1986-1992 Assistant Professor  
Department of Microbiology and Immunology  
University of California, San Francisco
- 1988-1992 Assistant Investigator  
Howard Hughes Medical Institute  
San Francisco, California, USA
- 1992-1995 Associate Professor  
Department of Microbiology and Immunology  
Associate Investigator  
Howard Hughes Medical Institute  
University of California, San Francisco
- 1995-1999 Professor and Vice Chair  
Department of Microbiology and Immunology  
Investigator  
Howard Hughes Medical Institute  
University of California, San Francisco
- 1999-2004 Professor and Director  
Institute of Biochemistry and Gene Center  
University of Munich, Germany
- 2004-present Director  
Max-Planck-Institute of Immunobiology  
Freiburg, Germany

## AWARDS

1982	Postdoctoral Fellowship from the European Molecular Biology Organization
1985	Special Fellowship from the Leukemia Society of America
1987	Leukemia Society of America Scholar
2000	Elected Member of the European Molecular Organization

## ADVISORY BOARD

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## EDITORIAL BOARDS

1990-present	<i>Molecular and Cellular Biology</i>
1992-present	<i>Genes and Development (European Editor 2002 – 2004)</i>
2002-present	<i>Immunological Reviews</i>

## RESEARCH TOPICS

Lymphocyte differentiation, gene regulation, Wnt signaling

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## Humanizing the mouse genome



The ability to replace mouse genes with their human equivalents using 'yeast artificial chromosome' technology provides a powerful new technique for studying the regulation and function of human genes.

The current human genome initiatives, which aim to map and ultimately sequence the complete human genome, are expected to produce a wealth of information about the chromosomal location and physical organization of many human genetic loci. However, while such studies can assign the transcribed region of a gene to particular DNA fragments of known position within a physical map of the human genome, by themselves they cannot fully define the extent of a gene — which includes regulatory sequences that can be some distance from the transcribed region — nor can they identify the gene's biological functions (though in some cases there will be hints from human mutations or sequence similarities to genes of known function). Thus, it is important that strategies and techniques are developed that will enable the functions of cloned human DNA sequences to be identified, preferably in a whole-animal context.

Ideally, such techniques will allow large stretches of human DNA to be transplanted into a 'model' mammalian host, in which the corresponding genes have been inactivated so that the host is dependent upon complementation of their biological functions by the introduced human genes. At present, the preferred host for such a strategy is the mouse, a small, prolific mammal that is amenable to genetic manipulation and that has been the subject of extensive genetic analysis for many years. Mouse embryonic stem (ES) cells have proved to be an effective tool for introduction of defined and selected genetic modifications into the mouse germline. When integrated into the mouse genome in ES cells, large DNA inserts can be evaluated for structural integrity, stability, copy number and integration into active chromatin sites, prior to their transmission into mice.

The development of yeast artificial chromosome (YAC) technology has permitted the cloning and genetic modification of DNA segments thousands of kilobases in size [1]. Such molecules are too large to be cloned by conventional cloning technologies, but when cloned between the YAC vector arms (Fig. 1) they can be stably maintained in yeast and are amenable to genetic manipulation by homologous recombination, which occurs efficiently in the host yeast cells.

YACs can be introduced into ES cells simply by isolating them from yeast cells and transfecting the ES cells with purified YAC DNA fragments. With YACs that are particularly large — as would be required to transmit

complex human loci in intact form — however, there is a danger that purification would shear the YAC DNA. This danger can be avoided — and the chances of preserving the structural identity of the YACs consequently increased — by an alternative approach, in which the YACs are shuttled into ES cells by fusion with yeast spheroplasts, shown previously to work with mammalian cell lines [2]. Questions can be raised about the effects of the fusion process itself, and the presence of co-transferred yeast genomic sequences, on the ability of ES cells to differentiate properly and to recolonize the mouse germline, but, as explained below, recent results suggest any such effects are negligible.

The faithful delivery of YACs carrying large fragments of human DNA into the mouse germline, using the cell fusion technique, was first reported last year [3] with a 670 kilobase (kb) YAC carrying a human X-chromosome fragment. The human hypoxanthine phosphoribosyl transferase (HPRT) gene, which occurs naturally on the human X-chromosome fragment carried by the YAC in this experiment, was used as a selectable marker following the polyethylene glycol-mediated fusion of HPRT-deficient ES cells with YAC-containing yeast spheroplasts. All the clones selected for expression of the HPRT gene were shown to contain the YAC, most (>90%) retaining the human insert completely intact, unrearranged and in a single copy.

About 40% of the selected clones retained both vector arms, indicating that the entire 670kb YAC had integrated into a mouse chromosome and suggesting that the vector arms could be used to carry markers for the selection of transformed ES cells. The co-integrated yeast

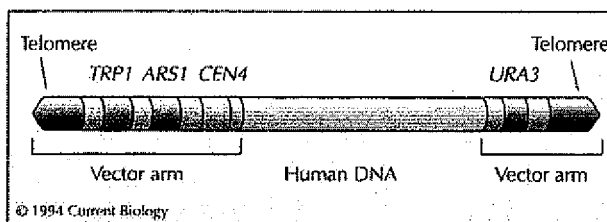


Fig. 1. Outline of the organization of a yeast artificial chromosome (YAC), which can be used to transfer large fragments of human DNA into the genomes of mouse ES cells and from there into mice. Both vector arms end in yeast telomeres, and one carries a centromere (*CEN4*) and a replication origin (*ARS1*); two genes (*TRP1* and *URA3*) act as selectable markers that stabilize the YACs in yeast cells.

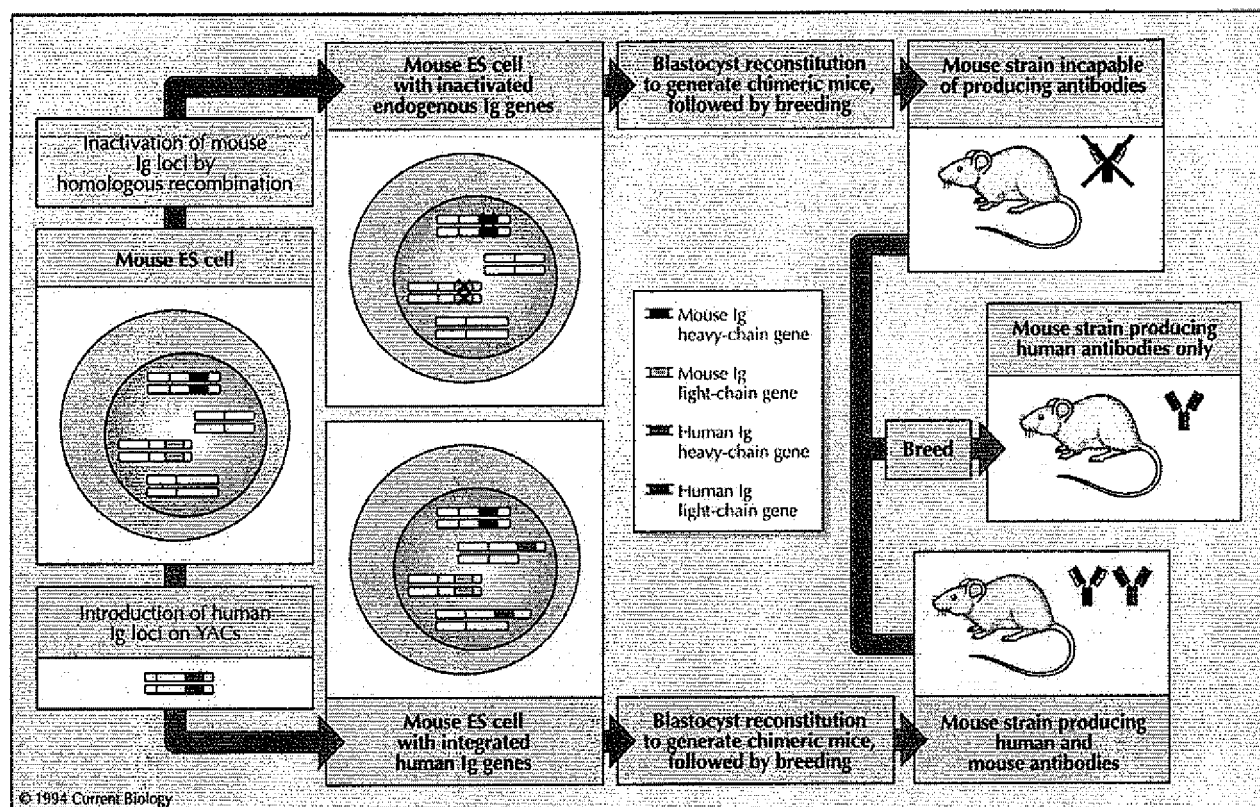


Fig. 2. An outline of the strategy my colleagues and I have recently used [8] to generate mice expressing human Ig genes, introduced on YACs, but not endogenous mouse Ig genes, inactivated by homologous recombination in ES cells.

genomic sequences did not affect the ability of ES cells to differentiate properly, either *in vitro* or *in vivo*, or to give rise to mice carrying the intact human X-chromosome fragment in their germline. The transmitted human sequences retained their function in the transgenic mice, as shown by the expression of human HPRT in all mouse tissues tested.

An alternative strategy for introducing YACs into ES cells is by transfecting the cells with gel-purified YAC DNA carried in lipid micelles, a technique known as lipofection. The feasibility of this approach has been demonstrated [4] with a 150kb YAC encompassing the mouse  $\alpha 1(I)$  collagen gene and flanking sequences, and also carrying a selectable neomycin-resistance gene. In 10% of the selected ES clones, the entire YAC was found to be intact and unrearranged. The YAC was transmitted faithfully into the mouse germline and the collagen transgene was found to be expressed at levels similar to that of the endogenous gene.

The demonstrations [3,4] that these two techniques — cell fusion and lipofection — both allow the efficient and reliable transfer of YACs into the mouse germline via ES cells has opened the way for their use to study the function and regulation of large and complex human genes, such as the  $\beta$ -amyloid precursor protein (APP) locus and the immunoglobulin (Ig) gene loci. The

human APP gene — 400kb long, composed of 18 exons and located on chromosome 21 — has been suggested to play a role in the pathogenesis of Alzheimer's disease and Down's Syndrome. In the hope that models for these human disorders might be developed from mice expressing the human APP gene in a properly regulated manner, two groups [5,6] have independently used the lipofection approach to transfer into ES cells a 650kb YAC carrying the entire human APP gene and flanking sequences, and have generated mice with the intact locus in their germline. Human APP transcripts [5,6] and proteins [5], with expression patterns mirroring those of endogenous mouse APP, were detected in the brain and peripheral tissues of these transgenic mice. The fidelity with which the human APP locus is regulated in the transgenic mice suggests that they may serve as a model system for studying the nature and development of Alzheimer's disease, and for testing therapeutic approaches to its prevention.

The genes encoding human Ig heavy and light chains each span over 2 megabases. In their germline configuration, these genes consist of separate segments encoding distinct parts of the Ig chains, such as the *V* *D* and *J* segments that encode the variable domains, and the *C* segments that encode the constant domains. The gene segments are sequentially joined by DNA rearrangements that accompany the development of mature B cells [7].

The ability to transfer these loci to the mouse genome using YAC technology has made it possible to assess the compatibility of human sequences with the mouse Ig gene recombination and expression machinery, and to study the relationship between the content and organization of the human sequences and their tissue-specific expression and function. Using the recently developed [8] strategy outlined in Figure 2, it is now possible to test the ability of human loci to substitute for their mouse equivalents — and in particular to generate mice that make fully human antibodies, which have a lower immunogenicity in humans and more desirable pharmacological properties than engineered mouse antibodies, and so are more suitable for human therapeutic applications.

Human genomic DNA fragments containing germline configuration V and C gene segments, including regulatory elements, of the Ig heavy-chain [8,9] and  $\kappa$  light-chain [8,10] loci have been successfully introduced into mouse ES cells using both fusion [8,10] and lipofection [9] techniques. The transformed ES cells were used to generate mice expressing individual human immunoglobulin genes [8,10]. In our work, the fusion technique worked with high efficiency, yielding a high frequency of clones carrying a single intact YAC [8]. A significant number of ES clones bearing an intact YAC but lacking detectable yeast DNA sequences were identified [8,10]; when present, however, the co-integrated yeast genomic DNA did not interfere with proper mouse development or with the assembly and expression of the human Ig genes [8].

The human Ig gene sequences were recognized, irrespective of their copy number or integration site, by the mouse machinery for gene recombination and expression. This was shown by their ability to undergo diverse rearrangements [8,10], to be expressed at significant levels and to prevent rearrangement of the mouse Ig genes (equivalent to the 'allelic exclusion' that normally ensures that B cells express only one Ig heavy-chain and one light-chain gene) [8]. In mice carrying the two types of human Ig YAC, both heavy and light Ig chains were expressed in B cells and assembled to generate membrane-bound or secreted, fully human antibodies [8]. When expressed in mice in which both endogenous heavy and  $\kappa$  light-chain genes had been inactivated by homologous recombination (Fig. 2), the human Ig genes were again rearranged and expressed properly [8], restoring B-cell differentiation and maturation. The treatment of these transgenic mice with antigen elicits an antigen-specific, human antibody response [8].

The human Ig gene products are thus properly expressed on the surface of B cells, which apparently interact normally with other cells of the mouse immune system. The mice generated a diverse antibody repertoire [8], reminiscent of adult human B cells. With the use of even larger DNA fragments from the human Ig loci, it should eventually be possible to recapitulate human antibody responses in mice. Such mice could be exploited to

elucidate the molecular mechanisms underlying the programmed assembly and expression of human antibody genes during different stages of development, in both normal and abnormal situations, such as autoimmune diseases and other disorders.

The results I have described establish the introduction of megabase-sized human loci into the mouse germline as a powerful approach to elucidating the function and regulation of very large or crudely mapped genes. It can be used to study complementation of recessive genetic disorders, to generate dominant mutations that may provide models of human diseases, such as those involving partial chromosome trisomy, as well as to identify distant *cis*-acting elements involved in the control of gene expression, or in regulatory processes such as genetic imprinting and X-chromosome inactivation. Furthermore, a strategy such as the one I have described for the mouse antibody genes can be applied towards humanization of other multi-gene loci, such as the major histocompatibility complex or the T-cell receptor loci, that govern different compartments of the mouse immune system. Mice in which the endogenous loci are replaced by the analogous human genes will be useful in gaining insights into the structure-function relationships of the human loci and their involvement in the evolution of the immune system.

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# Cloned transchromosomal calves producing human immunoglobulin

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Human polyclonal antibodies (hPABs) are useful therapeutics, but because they are available only from human donors, their supply and application is limited. To address this need, we prepared a human artificial chromosome (HAC) vector containing the entire unrearranged sequences of the human immunoglobulin (h/g) heavy-chain (*H*) and lambda (*λ*) light-chain loci. The HAC vector was introduced into bovine primary fetal fibroblasts using a microcell-mediated chromosome transfer (MMCT) approach. Primary selection was carried out, and the cells were used to produce cloned bovine fetuses. Secondary selection was done on the regenerated fetal cell lines, which were then used to produce four healthy transchromosomal (Tc) calves. The HAC was retained at a high rate (78–100% of cells) in calves and the h/g loci underwent rearrangement and expressed diversified transcripts. Human immunoglobulin proteins were detected in the blood of newborn calves. The production of Tc calves is an important step in the development of a system for producing therapeutic hPABs.

Despite the substantial need for hPABs to treat many diseases, the supply is limited to what can be obtained from human donors. Furthermore, the application of hPABs has been restricted because human donors cannot be hyperimmunized, that is, repeatedly boosted with antigen. Transgenic animals carrying h/g loci could provide a source of hPABs, especially targeted hPABs resulting from hyperimmunization with human pathogens or human molecules. Transgenic mice carrying h/g loci have been created<sup>1–6</sup> and are useful for the derivation of human monoclonal antibody therapeutics<sup>7,8</sup>.

Transgenic cattle carrying h/g loci could be useful for large-volume commercial production of hPABs. Transgenesis including gene targeting<sup>9–12</sup> in livestock has been reported; however, the procedures used are not suitable for transfer of the h/g loci<sup>13</sup> (1–1.5 Mb for each locus) because the maximum size of DNA that can be inserted is very limited (20–100 kb). Mammalian artificial chromosome (MAC) vectors<sup>14–17</sup> may be a better choice because of their large insert capacity. Thus far, there have been no reports of the transfer of MAC vectors in livestock. Furthermore, human microchromosomes are generally mitotically unstable in a foreign environment<sup>1,16,18</sup>. This could be a major obstacle in the production of transchromosomal cattle, which require a large number of cell divisions for full term development.

Another potential limitation of using cattle to produce hPABs is the difference in immunophysiology between cattle and humans<sup>19–22</sup>. In humans and mice, bone marrow is the major site of origin of all lymphocytes and the location of subsequent B-cell maturation. In contrast, spleen, rather than bone marrow, is the presumed site of B-cell origin and immunoglobulin rearrangement in bovine. Furthermore, because of a limited number of

functional V genes in bovine, gene conversion may be an important mechanism for the generation of diversity, especially for the light chain<sup>19,20</sup>. Gene conversion occurs in the ileal Peyer's patch, where B cells undergo proliferation and diversification. These differences could impede the functional rearrangement, diversification, and production of hIgs in cattle.

In this study, we developed a system for introducing heavy- and light-chain h/g loci into bovine by transferring a 10 Mb HAC vector carrying the loci into primary fibroblast cells and then producing cloned cattle from the Tc cells. We also evaluated the retention of the HAC through early gestation and the functional rearrangement, diversification, and expression of hIgs in the blood of Tc calves.

## Results

**HAC transfer into bovine fetal fibroblasts and nuclear transfer.** We constructed two HAC vectors ( $\Delta$ HAC and  $\Delta\Delta$ HAC), each carrying both h/g heavy-chain and  $\lambda$  light-chain loci, using a chromosome-cloning system<sup>17,23</sup> (Fig. 1). HAC vectors were introduced into bovine primary fetal fibroblasts from CHO clones using an MMCT system (Fig. 2). The life-span limits of bovine primary fibroblast cells required that complete antibiotic selection and DNA-based screening be avoided after MMCT to minimize cell divisions before nuclear transfer. Instead, we picked colonies on the basis of growth and morphology under selection and used them for nuclear transfer as quickly as possible. Nevertheless, the cells were useful only for a few days and could not be cryopreserved. Final selection was done after the rejuvenation and expansion of the cells during the growth of cloned fetuses. At 56–58 days, four

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**Table 1. Development of cloned embryos derived from Tc fibroblasts**

HAC	Nuclear transfer	Blastocysts (%) <sup>a</sup>	Blastocyst transfer	Recipients	Pregnant 40 days (%)	Pregnant 120 days (%)	Offspring (%) <sup>b</sup>
Δ	776	83 (18)	54	32	16 (50)	Fetal recovery	0
ΔΔ	833	122 (21)	56	28	13 (46)	Fetal recovery	1
Δ Regenerate	672	82 (17)	61	37	8 (22)	6 (16)	6 (16)

Fetal recovery was done between 56 and 119 days of gestation. Regenerated cells were produced from Tc fetuses recovered at 56–58 days of gestation.

<sup>a</sup>Percentage of blastocysts was calculated as the number of blastocysts per number of fused donor cell oocyte complexes. Average fusion rate in our laboratory was 70%.

<sup>b</sup>For ΔΔHAC nuclear transfers, one fetus was not recovered and developed to term producing a live healthy calf. For ΔHAC regenerated cells, six live calves were born; two from cell line 6032 did not survive past 48 h and the other four remain alive and healthy.

ΔHAC and two ΔΔHAC fetuses were recovered and fibroblast cell lines were regenerated, expanded, and cryopreserved for further analysis and nuclear transfer. Efficiency of development to the blastocyst stage, to 40 days of gestation, and to term is shown in Table 1 for first-generation and re-cloned cells.

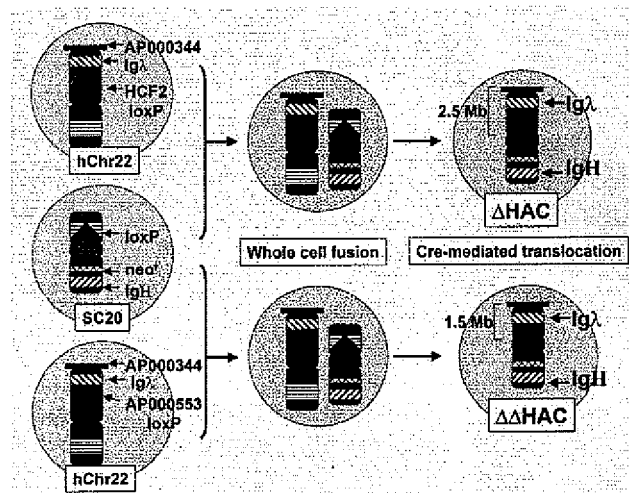
**Analysis of cloned Tc fetuses.** To examine whether the HAC vector was retained through early gestation and whether *hIg* loci could be functionally rearranged and expressed during early B-cell development in bovine, we analyzed cloned Tc fetuses collected between 56 and 119 days of gestation. Retention of the HACs in the fibroblast lines derived from six cloned Tc fetuses collected at 56–58 days and from an additional seven fetuses collected between 77 and 119 days was evaluated by G418 resistance (Fig. 3A) and by genomic PCR of human *IgH* and *Igλ* loci (Fig. 3B). Of the 13 fetuses, 9 were resistant to G418 (4 ΔHAC and 5 ΔΔHAC) and 8 showed the presence of both human *IgH* and *Igλ* loci. We evaluated five positive 77–119-day fetuses for expression and rearrangement of the *hIg* loci by reverse transcription-PCR (RT-PCR) analysis, followed by sequencing of the amplified products. Human *IgH* and *Igλ* genes were expressed (Fig. 3C) in all fetuses, predominantly in spleen, consistent with endogenous bovine *Ig*

expression (data not shown). The sequences showed evidence of functional V(D)J recombination (Fig. 3D). These results demonstrate retention of the HAC vector, functional V(D)J recombination, and expression of the *hIg* locus.

**Generation of cloned Tc calves.** For production of cloned Tc calves, the three regenerated ΔHAC

cell lines (5968, 6032, and 6045) were used for recloning. One male calf from cell line 6045 and five female calves from cell lines 5968 and 6032 were produced from 37 recipients (16%, Fig. 4A). Four calves survived and were healthy and phenotypically normal. Retention of the HAC vector was confirmed in all the calves by G418 selection (data not shown), genomic PCR (Fig. 4B), and fluorescent *in situ* hybridization (FISH) analyses (Table 2; Fig. 4C). FISH analysis indicated that the HAC was retained as an independent chromosome and that the proportion of cells retaining the HAC ranged from 78% to 100%. We observed no obvious differences in retention rates between peripheral blood lymphocytes (PBLs; 91%) and fibroblasts (87%). These data demonstrate that somatic-cell recloning strategies can be used to produce healthy cloned Tc calves and that the HAC vector can be stably maintained through the large number of cell divisions in bovine development.

**Human *Ig* gene expression and protein production in Tc calves.** To determine whether *hIg* loci were rearranged and expressed, we carried out RT-PCR analysis on PBLs. We observed expression of both human *IgH* and *Igλ* genes in the PBLs of all the calves (data not shown). The diversity of the human *IgH* and *Igλ* repertoire was determined by sequence analysis (Table 3). A representative set of the sequences showed a wide utilization of VH/VL, D, and JH/JL segments distributed over the loci. In the *IgH* transcripts, the frequent utilization of JH4 and of V segments from VH1 and VH3 was observed, similar to patterns in human<sup>24</sup>. Addition of non-germline nucleotides (N addition) and nucleotide deletion were also observed in both *hIgH* and *hIgλ* transcripts. These produced a high degree of diversification in the third complementarity-determining region of both the *hIgH* and *hIgλ* chains. Furthermore, *hIg* proteins were secreted at levels ranging from 13 ng/ml to 258 ng/ml (immunoglobulin expression is typically very low to undetectable in newborn calves<sup>25</sup>) in blood samples collected before colostrum feeding in five of the seven Tc calves. In the two calves in which *hIg* proteins were not detected, bovine

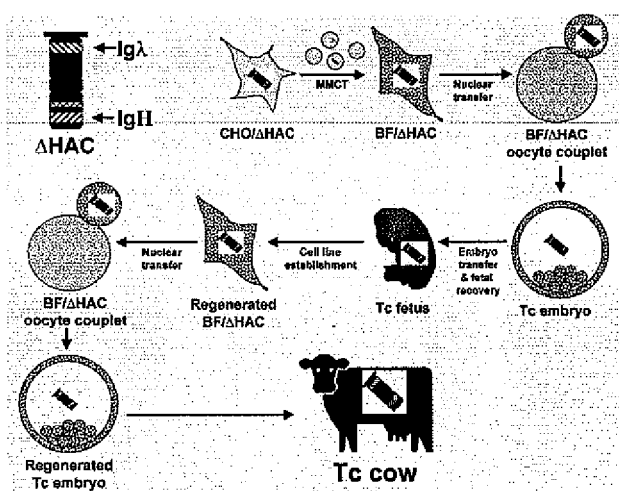


**Figure 1.** Diagram of ΔHAC and ΔΔHAC construction. In ΔHAC construction, the hChr22 fragment (hChr22), which contains the *Igλ* locus, was truncated at the AP000344 locus and a *loxP* sequence was integrated at the HCF2 locus. In ΔΔHAC construction, the *loxP* sequence on the hChr22 fragment was integrated at the AP000553 locus. DT40 cells containing the hChr22 fragments were fused with DT40 cells containing the SC20 fragment (SC20 is a fragment of hChr14 and contains the *IgH* locus). Cre-mediated translocation resulted in ΔHAC, which contained a 2.5 Mb hChr22 region, and ΔΔHAC, which contained a 1.5 Mb hChr22 region, each fused to the SC20 vector.

**Table 2. HAC retention in cloned Tc calves**

Calf number	Cell type	HAC positive/total (%)	Two signals/total (%)
50	PBL	50/50 (100)	6/50 (12)
50	Fibroblast	47/50 (94)	0/50 (0)
1064	PBL	46/50 (92)	0/50 (0)
1064	Fibroblast	34/39 (87)	3/39 (8)
1065	PBL	39/50 (78)	2/50 (4)
1065	Fibroblast	49/60 (82)	0/60 (0)
1066	PBL	47/50 (94)	1/50 (2)
1066	Fibroblast	43/50 (86)	0/50 (0)
Total	Combined	355/399 (89)	12/399 (3)

Retention rate was determined in both PBLs and fibroblasts in each calf by FISH analysis using human Col-I DNA as a probe.



**Figure 2.** Procedure for production of cloned Tc calves. Structure of  $\Delta$ HAC (upper left) is shown with hChr22 (green) and hChr14 (red) regions containing *IgH* and *IgL* loci. The centromere of the HAC is derived from the hChr14 fragment. The HAC was transferred from a CHO clone (CHO/ $\Delta$ HAC, light blue) into fetal bovine fibroblasts by means of a MMCT technique. Tc fibroblasts (BF/ $\Delta$ HAC, black patterned) and enucleated oocyte (yellow) couplets were fused, resulting in transfer of the fibroblast nucleus and formation of an embryo. The reconstituted Tc embryos were cultured *in vitro* to the blastocyst stage and then implanted into recipient cows. At ~60 days of gestation, Tc fetuses were recovered and Tc fibroblast cell lines were re-established (regenerated BF/ $\Delta$ HAC), evaluated, and used for further nuclear transfer. Regenerated Tc embryos were transferred to recipients to produce Tc calves.

immunoglobulin proteins were also not detected. These results demonstrate that the human *IgH* and *IgL* loci can be functionally rearranged and expressed in cattle.

## Discussion

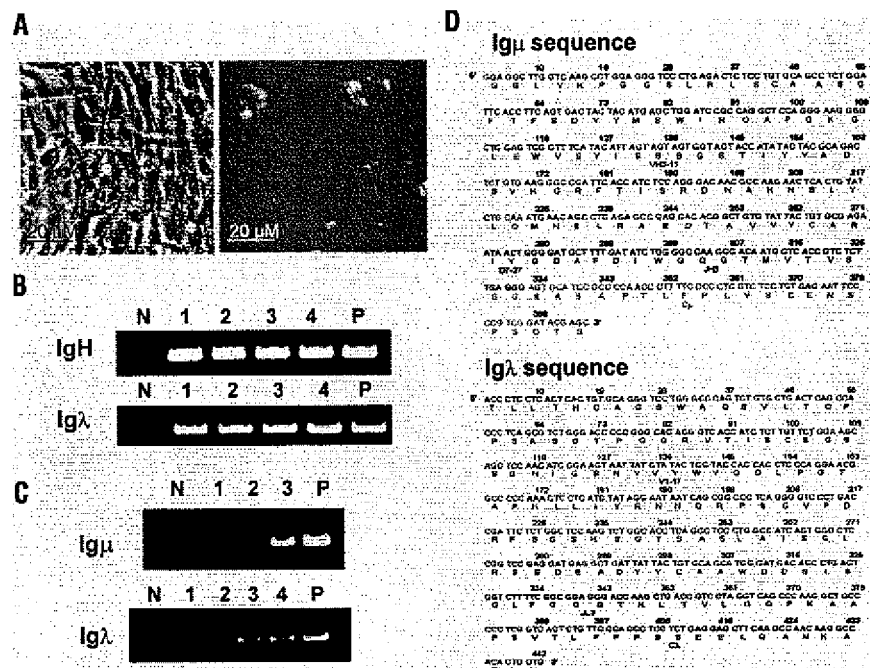
In this study, we demonstrate the production of healthy calves carrying a stable microchromosomal vector encoding human *IgH* and *IgL* loci, functional diversification of the hIg genes, and production of hIg protein. To accomplish this, several challenges needed to be addressed, including the lack of methods for introducing large foreign DNA sequences into cattle, the possible mitotic instability of a microchromosome vector, and the substantial differences in immune system development between cattle and humans.

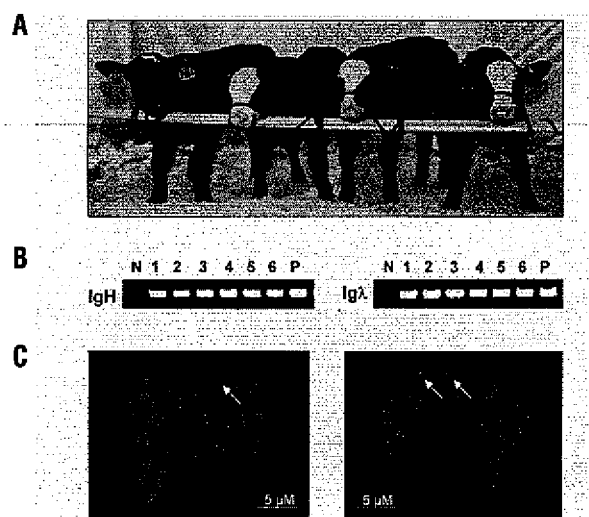
MMCT has been accomplished in mice using embryonic stem cells and chimera production followed by germline transmission from embryonic stem cell-derived germ cells<sup>1</sup>. Mouse embryonic

stem cells have the advantage of an unlimited life span, rapid growth in culture, a straightforward system for clonal propagation of transgenic colonies, and a high rate of contribution to the germ line in chimeras. Unfortunately, embryonic stem cells with similar properties have not been derived in other species, including cattle<sup>26</sup>. It was therefore necessary to use a nuclear transfer system with primary somatic cells<sup>27</sup> to produce Tc cattle. The limitation of this system is that primary fibroblasts, the cell type of choice, have a life span of ~35 population doublings<sup>28</sup>. After MMCT, our cells were capable of dividing for only about one week and could not be cryopreserved. Our approach to solving this problem was to carry out incomplete selection after MMCT and complete selection on rejuvenated cloned cell lines, and then produce calves by recloning cell lines. Recloning of transgenic somatic cells and production of offspring has been accomplished in cattle<sup>29</sup> with limited success. Our results indicate that rejuvenation of cell lines by recloning is a viable method for the production of large numbers of transgenic animals and the establishment of cryopreserved transgenic cell banks.

In mice, the SC20 HAC vector is retained in 70–80% of fibroblasts<sup>1</sup> and only 30–40% of PBLs (data not shown), revealing limitations in mitotic stability in a foreign environment. Because of the number of cell divisions required for bovine development, the retention rate of the HAC was expected to be low. However, the HAC was retained in all cloned calves at a very high rate, both in

**Figure 3.** Analysis of Tc fetuses. (A) G418 selection of regenerated Tc fibroblast line (left) and control nontransgenic fibroblasts (right). (B) Genomic PCR of *IgH* and *IgL* loci in Tc fetuses and controls. The three fetuses, 5968 (lane 1), 6032 (lane 2), and 6045 (lane 3) were derived from  $\Delta$ HAC fibroblasts; fetus 5580 (lane 4) was derived from  $\Delta$ HAC fibroblasts. As a control, a nontransgenic fetus (lane N) was recovered and evaluated. Human *IgH* and *IgL* loci were detected by PCR in all Tc fetuses and in a positive-control human liver DNA sample (lane P), but not in the negative control (lane N). (C) Rearranged and expressed human *IgH* and *IgL* transcripts amplified by RT-PCR from negative-control nontransgenic bovine spleen (lane N), from brain (lane 1), liver (lane 2), and spleen (lane 3 and 4) of cloned Tc fetus, and from positive-control human spleen (lane P). (D) A representative nucleotide and deduced amino acid sequence of human *IgH* and *IgL* transcripts amplified by RT-PCR from a cloned Tc fetus recovered at 99 days. In the *IgH* transcript, blue represents the variable region sequence (VH3-11), red represents the diversity region (D7-27), green represents the joining region (JH3) and orange represents the constant region. In the *IgL* transcript, blue represents the variable region sequence (VL1-17), red represents the joining region (JL3), and green the constant region sequence.





**Figure 4.** Analysis of cloned Tc calves. (A) Four cloned Tc calves: male calf (50) from cell line 6045 and female calves (1064, 1065, 1066) from cell line 5968. (B) Genomic PCR of *IgH* and *Igλ* loci in PBLs from cloned Tc calves and controls: calf 1064 (lane 1), 1065 (lane 2), 1066 (lane 3), 50 (lane 4), 1067 (lane 5), and 1068 (lane 6). Both human *IgH* and *Igλ* loci were detected by genomic PCR in all the Tc calves and positive-control human liver DNA (lane P), but not in a negative-control nontransgenic calf (lane N). (C) FISH analysis in metaphase chromosome spreads in a cell showing a single signal and a cell showing a double signal. Arrows indicate location of HACs (red) among surrounding bovine chromosomes (blue). A single HAC per cell is introduced and retained in most cells (left panel); however, improper separation of chromatids at cell division may result in some cells having two microchromosomes (right panel) and some not having a microchromosome.

fibroblasts (87%) and PBLs (91%). In our previous study<sup>17</sup>, the mitotically unstable hChr22 fragment was mitotically stabilized when its centromere was exchanged with that of the SC20 vector, suggesting that the mitotic stability of human microchromosomes might be affected by centromeric structure. In this study, the unexpectedly high mitotic stability of the HAC vector in bovine may be explained by greater compatibility of the human cen-

tromeric structure with factors regulating cell division in cattle as compared with mice. The high rate of HAC retention shows the utility of HAC vectors for stable introduction of foreign genetic elements into the genome of cattle.

We have shown that hIg loci can be functionally rearranged and expressed with substantial diversity in cattle despite the differences in immunophysiology between cattle and humans. Both broad usage of VDJ segments and N addition contributed considerably to hIg diversity in human IgH and Igλ transcripts in cloned Tc calves. Notably, in bovine Igλ transcripts, N addition is rarely observed (data not shown). These results indicate that immunoglobulin gene diversification may be more related to genetic sequence than to the environment in which diversification occurs.

**Table 3. Repertoire analysis of human immunoglobulin heavy- and λ-chain transcripts in cloned Tc calves**

Human μ nucleotide sequences				
VH	N	DH	N	JH
6-1	0	D5-24	3	JH3
TACTGTGCA		AGAGATG	AGA	ATGCTTTTGATGTC
3-33	8	D6-13	3	JH4
ATTACTGTGCGA	AGAACAAA	ATAGCAGCAGCTGGTAC	GAT	CTTTGACTACT
3-15	4	D6-19	4	JH1
ACTGTACCACAGA	TCTG	ATAGCAGTGGCTGGTAC	TGGG	TACTTCCAGCA
3-66	2	D2-2	0	JH3
TACTGTGCGAG	TC	GTAGTACCAGCTGCTAT		GATGCTTTTGATGTCT
3-21	6	D2-21	8	JH4
TTACTGTGCGAG	TTTTGG	GTGGTGGT	CACATTGA	GACTACTGGGG
4-39	8	D3-10	3	JH4
ACTGTGCGAGACA	TGAAAAAC	TTCGGGGAGTTAT	AAT	CTACTGGGGCC
1-69	7	D6-13	1	JH4
TTACTGTGCGAG	GGGGATG	GCAGCAGCTGGTAC	C	GACTACTGGGGC
1-8	0	D2-2	12	JH2
ACTGTGCGAGAG		ATTGTAGTAGTACCAGCTGC	CAAGATCGTAAG	TGGTACTTCGAT
1-18	0	D5-24	15	JH4
TTACTGTGC		GAGATGG	GTTTTTGATCCCCAG	TTTGACTACTGG
3-20	4	D7-27	1	JH3
TCACTGTGCGAGAA	TTTT	ACTGGGGA	T	GATGCTTTTGATGTCT
Human λ nucleotide sequences				
VL	N	JL		
1-17	2 (TT)	JL3	TCGGCGGAGGG	
2-13	0	JL2	GGTATTCGGCGGAGG	
1-19	0	JL1	TCTTCGGAAGTGGG	
5-2	2 (TA)	JL3	GTTTCGGCGGAGAG	
1-7	1 (C)	JL3	TCGGCGGAGGGA	
2-13	0	JL1	TATGTCTTCGGAAGTG	
2-1	0	JL1	TATGTCTTCGGAAGTG	
1-2	1 (G)	JL1	ATGTCTTCGGAAGTG	
1-4	2 (GT)	JL3	TCGGCGGAGG	
1-4	0	JL1	GGAAGTGGGA	

Human μ- and λ-specific mRNAs were amplified by RT-PCR, cloned, and sequenced. Nucleotide sequences of VDJ junctions of each of ten independent μ and λ clones are shown, divided into Vh/Vλ, D, Jh/Jλ, and N segments, as identified by homology to published germline sequences (Ig-BLAST).

Several challenges remain before our system can be used for large-scale production of hPABs. Because Tc cattle retain the bovine Ig loci, expression of bovine antibody is expected to dominate over that of human antibody. We have observed this previously in Tc mice in which the murine Ig loci were not inactivated (data not shown). Furthermore, chimeric antibodies containing combinations of human and bovine heavy- and light-chain proteins are expected to be present. Therefore, methods of reducing bovine Ig expression are probably needed before commercial production of hPABs can proceed in Tc cattle.

A Tc bovine-based system for producing therapeutic hPABs would have several advantages: (i) cattle could be hyperimmunized with essentially any human pathogen or human molecule; (ii) cattle produce very large quantities of antibodies; (iii) large numbers of antigens could be evaluated quickly because one line of genetically modified cattle could be used for all antigens; and (iv) scale-up of antibody production would be as straightforward as immunizing additional cows. Therapeutic hPABs produced in a Tc bovine-based system may have broad application in the treatment and prevention of infectious disease (including antibiotic resistant infections), autoimmune disease, and cancer.

## Experimental protocol

**Construction of HAC vectors.** HACs were constructed using a previously described chromosome-cloning system<sup>17,23</sup> (Fig 1). Briefly, for the construction of  $\Delta$ HAC, the previously reported hChr22 fragment (hChr22) containing a *loxP* sequence integrated at the *HCF2* locus was truncated at the *AP000344* locus by telomere-directed chromosomal truncation. Next, cell hybrids were formed by fusing the DT40 cell clone containing the hCF22 with a DT40 cell clone containing the stable and germline-transmissible human microchromosome vector SC20. The resulting DT40 cell hybrids contained both hChr fragments. The DT40 hybrids were transfected with a Cre recombinase expression vector to induce Cre-*loxP*-mediated chromosomal translocation between hChr22 and the SC20 vector. The stable transfectants were analyzed using nested PCR to confirm the cloning of the 2.5 Mb hChr22 region into the *loxP*-cloning site in the SC20 vector ( $\Delta$ HAC).  $\Delta$ HAC was constructed using the same chromosome cloning system except that the *loxP* sequence on hChr22 was integrated into the *AP000553* locus, creating a 1.5 Mb insert upon Cre-*loxP*-mediated translocation.

**HAC vector transfer into bovine fetal fibroblasts.** Bovine fetal fibroblasts were cultured in  $\alpha$ -MEM (Life Technologies, Rockville, MD) medium supplemented with 10% (vol/vol) FCS (Life Technologies) at 37°C and 5% CO<sub>2</sub>. Microcells were purified from the CHO clone retaining the  $\Delta$ HAC or  $\Delta$ HAC as described previously<sup>17</sup>. Bovine fetal fibroblasts were fused with microcells using polyethylene glycol (PEG 1500, Roche, Nutley, NJ), and the fused cells were selected under 700  $\mu$ g/ml of G418 (Life Technologies) for 10–14 days. The G418-resistant clones were picked and used for nuclear transfer.

**Nuclear transfer.** The nuclear transfer procedure was carried out essentially as described previously<sup>27,28</sup>. *In vitro*-matured oocytes were enucleated

–18–20 h post maturation. Cytoplasm-donor cell couplets were fused using a single electrical pulse of 2.4 kV/cm for 20  $\mu$ s (Electrocell Manipulator 200, Genetronics, San Diego, CA). At 30 h, post maturation reconstructed oocytes were activated with calcium ionophore (5  $\mu$ M) for 4 min (Cal Biochem, San Diego, CA) and 10  $\mu$ g cycloheximide and 2.5  $\mu$ g cytochalasin D (Sigma) as described earlier<sup>28</sup>. After activation, cloned embryos were placed in culture in four-well tissue culture plates, containing irradiated mouse fetal fibroblasts and 0.5 ml of ACM culture medium covered with mineral oil (Sigma) and incubated at 38.5°C in a 5% CO<sub>2</sub> in air atmosphere. On day 4, 10% (vol/vol) FCS (Life Technologies) was added to the culture medium. On days 7 and 8, embryos were transferred into synchronized recipients. All animal work was done following a protocol approved by the Trans Ova Genetics (Sioux Center, IA) institutional animal care and use committee.

**Genomic PCR analysis.** Genomic DNA was extracted from Tc fetuses at 56–119 days of gestation or from cloned newborn calves and subjected to PCR using primers IGHV3 for the human IgH locus and IGLC for the human IgL locus, as described previously<sup>17</sup>.

**RT-PCR and repertoire analyses.** Total RNA was recovered from spleen, liver, and brain of Tc fetuses or from PBLs of Tc calves. RT-PCR was carried out as described previously<sup>30</sup>. For human IgH transcripts, VH1/5 BACK, VH3 BACK, and VH4 BACK were used as a 5' primer and C $\mu$ -2 was used as a 3' primer. For human IgL transcripts, V $\lambda$ 1LEA1, V $\lambda$ 2MIX, and V $\lambda$ 3MIX were used as a 5' primer and C $\lambda$ MIX was used as a 3' primer. The amplified cDNAs were subcloned by using a TA cloning kit (Invitrogen, San Diego, CA) and sequenced using a DNA autosequencer (ABI3700 Sequencer, GlaxoWellcome, Herts, United Kingdom).

**FISH analysis.** Digital image analysis was done using the Mac Probe system (Applied Imaging, Santa Clara, CA). HAC painting was done using digoxigenin-labeled (Boehringer Ingelheim, Ridgefield, CT) human Cot-1 DNA as a probe, and the digoxigenin signal was detected with an anti-digoxigenin-rhodamine complex that fluoresced red. DAPI (Sigma) was used for background staining. Standard chromosome and FISH protocols were carried out as described<sup>31</sup>.

**ELISA analysis for human antibody.** Plasma samples were obtained from Tc calves before they were fed colostrum and human Ig levels were determined by solid-phase ELISA. The assay used a bovine anti-human immunoglobulin as the capture antibody and an HRP-labeled sheep anti-human immunoglobulin as the detecting antibody. Amounts of human immunoglobulin >10 ng/ml were reliably detected by this assay.

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## Competing interests statement

The authors declare competing financial interests: see the Nature Biotechnology website (<http://www.nature.com/naturebiotechnology>) for details.

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# A Tissue-specific Transcription Enhancer Element Is Located in the Major Intron of a Rearranged Immunoglobulin Heavy Chain Gene

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## Summary

We have studied the DNA sequences required for high level expression of a cloned heavy chain immunoglobulin gene stably introduced into mouse myeloma cells by DNA transfection. We found that DNA sequences derived from the germ line  $J_H-C_H$  region are required for accurate and efficient transcription from a functionally rearranged  $V_H$  promoter. Similar to viral transcriptional enhancer elements, these cellular sequences stimulate transcription from either the homologous  $V_H$  gene segment promoter or a heterologous SV40 promoter. They are active when placed on the 5' or 3' side of the rearranged  $V_H$  gene segment and they function when their orientation is reversed. However, unlike viral enhancers, the Ig gene enhancer appears to act in a tissue-specific manner, since it is active in mouse B cells but not in mouse fibroblasts. The nucleotide sequence of the Ig enhancer region contains repeating elements that closely resemble sequence elements found in many viral enhancers. We discuss the possible role of tissue-specific transcription in cell differentiation and malignant transformation.

## Introduction

Molecular analyses of immunoglobulin (Ig) genes established that an Ig polypeptide chain is encoded in multiple gene segments scattered along a chromosome of the germ line genome and that these gene segments must be brought together to form a complete Ig gene active in B lymphocytes (For a review see Tonegawa, 1983). This somatic assembly of Ig gene segments is achieved by a series of developmentally-controlled recombination events that occur during the differentiation of B cells.

Two types of recombination occur: V-J or V-D-J joining and switch recombination. The V-J and V-D-J joinings are essential for the formation of the DNA sequence coding for the variable (V) region of the light and heavy chain, respectively, while switch recombination replaces the constant (C) region-coding sequence of the heavy chain of one class (usually  $\mu$ ) with another ( $\gamma$ ,  $\epsilon$ ,  $\alpha$ , etc.). The exact

timing of these recombination events in the course of B cell development has not been determined, but both V-J and V-D-J joining occur before the cell encounters antigens.

One of the most important functions of the V-J and V-D-J joinings is to create complete Ig genes with a diverse set of V region-coding DNA sequences from a limited number of the gene segments carried in the germ line genome (Tonegawa, 1983). Another important function of V-J and V-D-J joining events is in the control of the expression of Ig genes during B cell differentiation. On the basis of the fine structural analysis of Ig genes, it is clear that these joining events are prerequisite for the synthesis of a complete Ig chain: there is no evidence that any unrearranged germ line Ig gene segment can directly participate with its coding capacity in the synthesis of a complete, functional Ig chain (Brack et al., 1978; Bernard et al., 1978; Sakano et al., 1979; Max et al., 1979; Early et al., 1980; Sakano et al., 1980). In fact, it has been shown that in a myeloma cell the RNA transcript of an unrearranged  $V_H$  segment is no more than 0.1 copy per gene, a level at least four orders of magnitude lower than that of the transcript from the rearranged, expressed  $V_H$  segment present in the same cell (Mather and Perry, 1981).

Both the V-J and V-D-J joining events alter the sequence configurations in the 3' region of the germ line V gene segment, but the 5' flanking region of the V gene segment, where the transcription promoter and other controlling elements reside, is unaffected by the rearrangement (Bernard et al., 1978 and Clarke et al., 1982). Although a few nucleotide differences have been found between the germ line and somatic sequences in the 5' flanking region of V gene segments (Sakano et al., 1980), these base substitutions are by-products of the somatic mutation events whose physiological role is to diversify the V-coding sequences (Bernard et al., 1978; Weigert and Riblet, 1976; Selsing and Storb, 1981). These base changes are not systematic and therefore are thought to have no bearing on the control of Ig gene expression (Clarke et al., 1982).

A possible explanation of how the downstream sequence might confer transcriptional competence to the rearranged V gene segment promoter is through transcriptional enhancement. Although the mechanism of this phenomenon is unknown, specific viral DNA sequence elements have been described (Banerji et al., 1981; de Villiers et al., 1981; Levinson et al., 1982) which enhance viral or recombinant cellular gene transcription. Because such enhancer elements can activate transcription from promoters which are located either upstream or downstream, and more than 1 kb away, it is possible that an analogous element might be located near the C gene segment. The observations that the C gene segments, in contrast to the V gene segments, are transcriptionally active in lymphoid cells in the absence of rearrangement support this hypothesis. In this case, promoter-like sequences upstream of the C gene segments are utilized for transcription but the

transcripts are degraded in the nuclei (Kemp et al., 1980; Van Ness et al., 1982).

The recent technical advances for introducing cloned immunoglobulin genes into lymphoid cells (Oi et al., 1983; Rice et al., 1983) have made it possible to study the structure-function relationship between specific DNA sequences and gene expression in these cells. We describe an enhancer element in the major intron of a rearranged  $\gamma_{2b}$  heavy chain gene. This sequence is located between the  $J_H$  region and the switch-recombination site utilized in myeloma MOPC 141, i.e., it is derived from sequences upstream of the germ line  $C_H$  gene segment.

## Results

### High Level Expression of the Heavy Chain Gene Introduced into Myeloma Cells

We previously reported that the functionally rearranged immunoglobulin heavy chain ( $\gamma_{2b}$ ) gene from MOPC 141 tumor cells (Sakano et al., 1980) can be accurately expressed at a low level in transfected mouse L cells (Gillies et al., 1983). For the studies presented here, we subcloned the same  $\gamma_{2b}$  gene fragment into plasmid pSV2gpt (Mulligan and Berg, 1980), transfected the mouse myeloma line, J558L, and selected for gpt gene activity by resistance to mycophenolic acid. This J558L line has lost the ability to express the endogenous immunoglobulin heavy chain gene but continues to synthesize a  $\lambda$  light chain. Furthermore, J558L has been shown to have a relatively high transformation frequency ( $>10^{-4}$ ) when pSV2gpt vectors containing light chain genes are used for transfection (Oi et al., 1983).

Using a modified protocol for protoplast fusion (see Experimental Procedures), we found that plasmid pSV- $\gamma_{2b}$ VC (Figure 1) transforms J558L cells at a frequency of greater than  $10^{-3}$ . This high frequency made it possible to use pools of independently-derived clones of gpt transformants to compare the expression of plasmids containing defined deletions with that of the parental plasmid pSV- $\gamma_{2b}$ VC. The advantage of this method is that the resulting cell lines represent several independent integration events (required for transformation), therefore the level of heavy chain gene expression in a given pool should reflect the average level of the individual clones. Thus the possible effect of the site of integration on the expression of the transfected gene is minimized.

Cell lines obtained by transfection with plasmid pSV- $\gamma_{2b}$ VC and selection for gpt expression (growth in the presence of mycophenolic acid) were found to express high levels of  $\gamma_{2b}$  heavy chain (Figure 2A, lanes 2-5). These levels of expression of the exogenous  $\gamma_{2b}$  genes are estimated to be about 20% of that of the endogenous  $\gamma_{2b}$  gene in MOPC 141. Apparently, this heavy chain can form an immunoglobulin molecule with the  $\lambda$  light chain of myeloma J558L, because the light chain was immunoprecipitated from cell extracts with antiheavy chain antisera and equimolar amounts of heavy and light chain were secreted into the culture medium (Figure 2A, lane 14).

### A Deletion of Part of the Major Intron Abolishes the High Level Expression of the Heavy Chain Gene

Deletion mutants of the parental plasmid were constructed to test whether the removal of specific noncoding DNA sequences would affect the expression of the  $\gamma_{2b}$  gene in J558L cells. Because deletions between the VDJ and  $C_H$  exons of an Abelson murine leukemia virus-transformed cell line have been correlated with decreased heavy chain production (Alt et al., 1982), we constructed mutant plasmids with deletions in this region. Two such plasmids, pSV- $\gamma_{2b}$ 3'RD1 and pSV- $\gamma_{2b}$ 3'RD2 contain overlapping deletions around the unique Eco RI site of the parental plasmid pSV- $\gamma_{2b}$ VC (Figure 1). These three plasmids were introduced into J558L myeloma cells and the expression of the  $\gamma_{2b}$  heavy chain gene in stably transformed cells was compared.

Cell lines obtained by transfection with plasmid pSV- $\gamma_{2b}$ 3'RD1 synthesized high levels (no less than half of the wild type level) of  $\gamma_{2b}$  heavy chain (Figure 2A, lanes 6-9) and secreted immunoglobulin (Figure 2A, lane 15). In contrast, four cell lines obtained by transfection with plasmid pSV- $\gamma_{2b}$ 3'RD2 synthesized only low levels (about 5% of the wild type level) of heavy chain (Figure 2A, lanes 10-13). The same results were obtained when subclones of

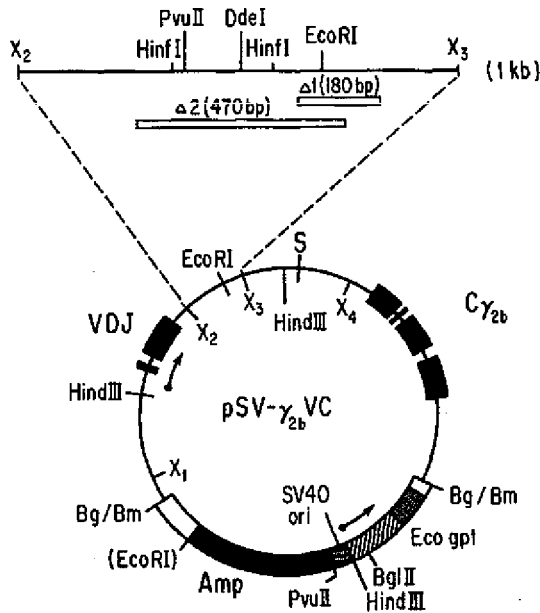
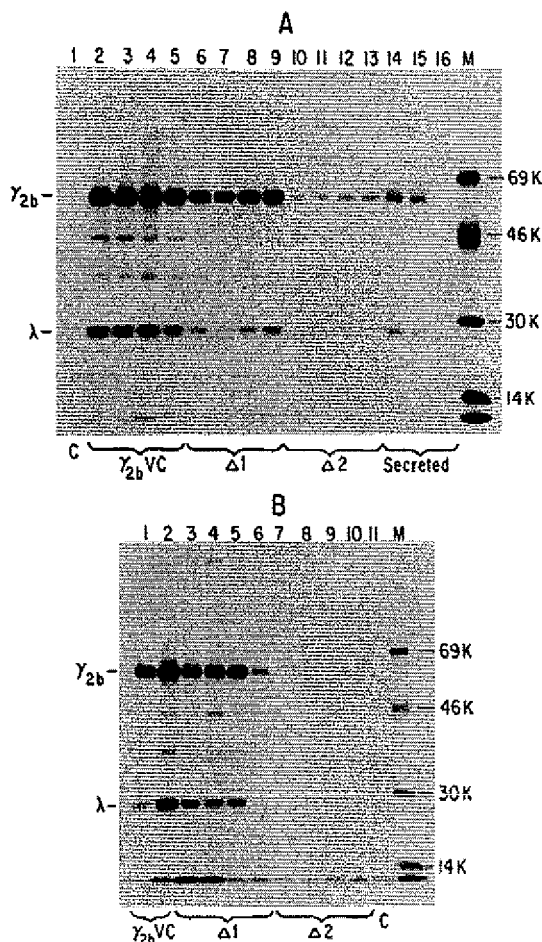


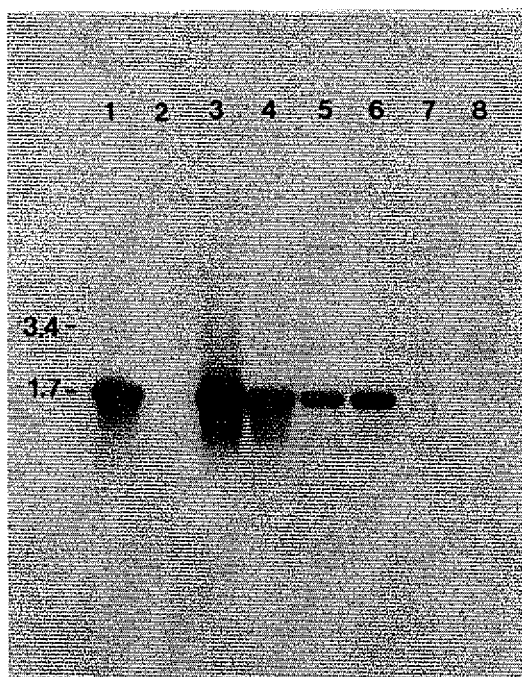
Figure 1. Partial Restriction Map of Plasmid pSV- $\gamma_{2b}$ VC

A 9 kb Bgl II fragment was inserted into the Bam HI site (indicated by Bg/Bm) of plasmid pSV2gpt. The Ecogpt gene (wide stripes) is flanked by SV40 sequences (thin stripes) including the origin (ori) of replication and mRNA start site (arrow). The  $\gamma_{2b}$  gene (narrow line) contains VDJ and  $C_{\gamma_{2b}}$  exons (solid boxes) and a mRNA start site (arrow) about 30 bp upstream of the VDJ coding sequence (Gillies and Tonegawa, 1983). The switch recombination (S) site is also shown. The DNA segments deleted in plasmids pSV- $\gamma_{2b}$ 3'RD1 and pSV- $\gamma_{2b}$ 3'RD2 are shown in linear form above the circular map. The sizes of the deletions, as determined by restriction analysis, are indicated. The exact locations of these deletions are shown in Figure 7.



**Figure 2. Expression of  $\gamma_{2b}$  Heavy Chain Protein in Transfected Cells**  
Transfected cell lines were labeled with  $^{35}\text{S}$ -methionine and cell extracts were analyzed as described in Experimental Procedures. Immunoprecipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. (A) Four transfected lines (pools of individual clones) were analyzed for each plasmid tested. Lane 1: control J558L cells; lanes 2-5: cells transfected with plasmid pSV- $\gamma_{2b}$ VC; lanes 6-9: cells transfected with plasmid pSV- $\gamma_{2b}$ 3'RA1; lanes 10-13: cells transfected with plasmid pSV- $\gamma_{2b}$ 3'RA2. Secreted proteins from cells transfected with plasmid pSV- $\gamma_{2b}$ VC (lane 14), plasmid pSV- $\gamma_{2b}$ 3'RA1 (lane 15), and plasmid pSV- $\gamma_{2b}$ 3'RA2 (lane 16) were immunoprecipitated and analyzed on the same gel. (B) Cell lines subcloned from the transfected cell lines were tested for  $\gamma_{2b}$  heavy chain protein synthesis as in (A). The plasmids used for transfection are indicated below the autoradiogram. Control (C) cell extract is shown in lane 11. The positions of the  $\gamma_{2b}$  heavy chain and  $\lambda$  light chain (synthesized in J558L cells but not immunoprecipitated in the absence of  $\gamma_{2b}$  heavy chain) are indicated.

each pool were tested for  $\gamma_{2b}$  heavy chain expression (Figure 2B), although more variation was observed in the level of expression between individual clones. Nonetheless, these results strongly suggest that DNA sequences deleted in plasmid pSV- $\gamma_{2b}$ 3'RA2, but still present in pSV- $\gamma_{2b}$ 3'RA1, are essential for the high level expression of heavy chain genes in myeloma cells.



**Figure 3. Northern Gel Blotting Analysis of Transfected Cell RNA**  
Total cell RNA (10  $\mu\text{g}$  per lane) was electrophoresed on a denaturing agarose gel, transferred to nitrocellulose, and hybridized to nick-translated  $\text{C}\gamma_{2b}$  probe. RNA (2.5  $\mu\text{g}$ ) from MOPC 141 cells (lane 1) and J558L cells (lane 2) were also analyzed. Two cell lines transfected with plasmid pSV- $\gamma_{2b}$ VC (lanes 3 and 4), plasmid pSV- $\gamma_{2b}$ 3'RA1 (lanes 5 and 6), and plasmid pSV- $\gamma_{2b}$ 3'RA2 (lanes 7 and 8) are shown. The positions of the secreted (1.7 kb) and membrane forms (3.4 kb) of  $\gamma_{2b}$  mRNA are indicated.

### The Reduced Expression of the $\gamma_{2b}$ Gene Is at the Level of RNA

The steady-state level of  $\gamma_{2b}$  mRNA in transfected cell lines was analyzed by Northern gel blotting and hybridization with the  $\text{C}\gamma_{2b}$  probe. It should be noted that the  $\gamma_{2b}$  heavy chain gene used in these studies does not contain the exons coding for the membrane form of  $\gamma_{2b}$  (Gillies and Tonegawa, 1983) and thus the only species of mRNA expected in transfected cells is the secreted form (1.7 kb).

As seen in Figure 3, cell lines transfected with plasmid pSV- $\gamma_{2b}$ VC (lanes 3 and 4) and plasmid pSV- $\gamma_{2b}$ 3'RA1 (lanes 5 and 6) contain high levels of the secreted form of  $\gamma_{2b}$  mRNA. The cell lines transfected with plasmid pSV- $\gamma_{2b}$ 3'RA2 (Figure 3, lanes 7 and 8) contained much lower levels of  $\gamma_{2b}$  mRNA of the correct size, in agreement with the decreased level of  $\gamma_{2b}$  heavy chain protein (Figure 2). The additional RNA bands seen in lanes 7 and 8 (Figure 3) also contain  $\gamma_{2b}$  sequences but appear to be read-through products of the *Ecogpt* gene. Data presented below support this explanation, as opposed to the idea that the intron deletion has a deleterious effect on RNA splicing and results in low levels of translatable  $\gamma_{2b}$  mRNA.

### Plasmid Copy Number in Transfected Cell Lines

DNA from transfected cells was analyzed by Southern gel blotting to determine the plasmid copy number and its possible effect on the level of  $\gamma_{2b}$  mRNA. When a pSV2gpt plasmid DNA probe was used for hybridization, a striking difference in plasmid copy number was found (Figure 4). Two prominent bands, corresponding to the two large Hind III fragments (6.2 kb and 5.0 kb) common to all the plasmids, are detected with this probe (seen best in Figure 4, lanes 6 and 7). Clearly, the DNA sequences deleted in plasmid pSV2- $\gamma_{2b}$ 3'RA2 (those required for the high level expression of  $\gamma_{2b}$  mRNA) have a dramatic effect on the number of copies of plasmid required for transformation to the *gpt*<sup>r</sup> phenotype. When these sequences are present, as they are in plasmids pSV- $\gamma_{2b}$ VC and pSV- $\gamma_{2b}$ 3'RA1, a low copy number is sufficient for *gpt* transformation (Figure 4, lanes 2-5). In the absence of these sequences, the copy number is increased at least 20-fold (Figure 4, lanes 6 and 7), presumably to compensate for a comparable decrease in *gpt* mRNA transcription.

Two conclusions can be made from these results: one, the DNA sequences required for the high level expression of  $\gamma_{2b}$  mRNA also increase the level of expression from the heterologous SV40 promoter at least 20 times; two, the level of RNA transcribed from the V gene segment promoter is decreased about 400 times per gene copy in the absence of this DNA sequence. This calculation is based on the observed decrease by a factor of 20 in  $\gamma_{2b}$  gene expression as a result of the 3'RA2 deletion, and the fact that this decreased level is likely the result of the transcription of at least 20 times as many gene copies.

### DNA Sequences Located in the $\gamma_{2b}$ Gene Intron Enhance Expression in an Orientation-independent and a Position-independent Manner

The DNA sequences defined as viral enhancer elements have been shown to stimulate the transcription of homologous or heterologous promoters either upstream or downstream, and in either orientation with respect to the direction of transcription (Moreau et al 1981; Wasyluk et al, 1983). In order to test whether the sequences located in the major intron of the  $\gamma_{2b}$  gene (and all other heavy chain genes) behave similarly, we constructed a plasmid with most of the intron sequences deleted. We then inserted a 1 kb Xba I fragment ( $X_{2/3}$ ) containing those intron sequences with potential enhancer activity into either of two sites in either of the two orientations. The first corresponds to the original position of this fragment in the parental plasmid (as part of the VDJ- $\gamma_{2b}$  intron) and the second is approximately 1.4 kb upstream (on the 5' side of the V gene segment). Four plasmids were obtained which contained the  $X_{2/3}$  fragment in the normal or reversed orientation, either upstream or downstream of the mRNA start site (see Figure 5A).

Cell lines obtained by transfection with the plasmids just described were analyzed for the expression of  $\gamma_{2b}$  heavy chain. As seen in Figure 5B, cells transfected with plasmid pSV- $\gamma_{2b}$  $\Delta X_{2/3}$  (with most of the intron deleted) did not

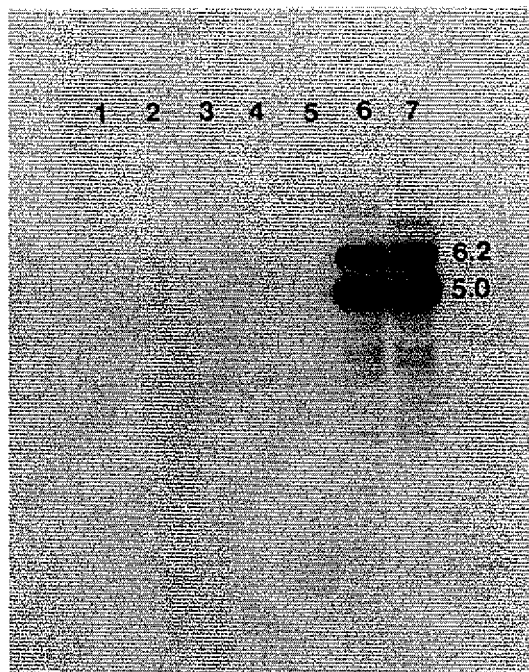


Figure 4. Southern Gel Blotting Analysis of Plasmid DNA Sequences in Transfected Cell Lines

High molecular weight DNA (10  $\mu$ g per lane) from J558L cells (lane 1), or cell lines transfected with plasmid pSV- $\gamma_{2b}$ VC (lanes 2 and 3), plasmid pSV- $\gamma_{2b}$ 3'RA1 (lanes 4 and 5), or plasmid pSV- $\gamma_{2b}$ 3'RA2 (lanes 6 and 7) was digested with Hind III, electrophoresed on a 0.8% agarose gel, transferred to nitrocellulose, and hybridized to nick-translated pSV2gpt DNA. The positions of the two Hind III fragments (6.2 kb and 5.0 kb), common to all the transfected plasmid DNAs (both of which hybridize to the pSV2gpt probe), are indicated.

synthesize significant levels of  $\gamma_{2b}$  protein (lanes 3 and 4). The insertion of the  $X_{2/3}$  fragment into the intron site (the normal position of this fragment) restored the expression of  $\gamma_{2b}$  protein in both the normal (Figure 5B, lanes 5 and 6) or reversed (Figure 5B, lanes 7 and 8) orientations. Similarly, insertion of the same fragment upstream of the V gene segment (on the 5' side of the transcriptional promoter) in either the normal (Figure 5B, lanes 9 and 10) or the reversed (Figure 5B, lanes 11 and 12) orientation also restored the expression of  $\gamma_{2b}$  protein to normal levels.

These results clearly demonstrate that the intron sequences deleted in the 3'RA2 mutant plasmid have a direct effect on transcription in a manner that is analogous to the viral enhancers. They also show that the enhancer function does not require the expression of these sequences in the  $\gamma_{2b}$  gene primary transcript, because movement of the  $X_{2/3}$  fragment outside of the transcription unit (i.e., the Xba I<sub>1</sub> site) had no effect on its ability to function.

### Tissue Specificity of the Immunoglobulin Enhancer Element

The rearranged  $\gamma_{2b}$  gene used in these studies is also accurately transcribed in mouse fibroblasts (Ltk<sup>-</sup> cells)

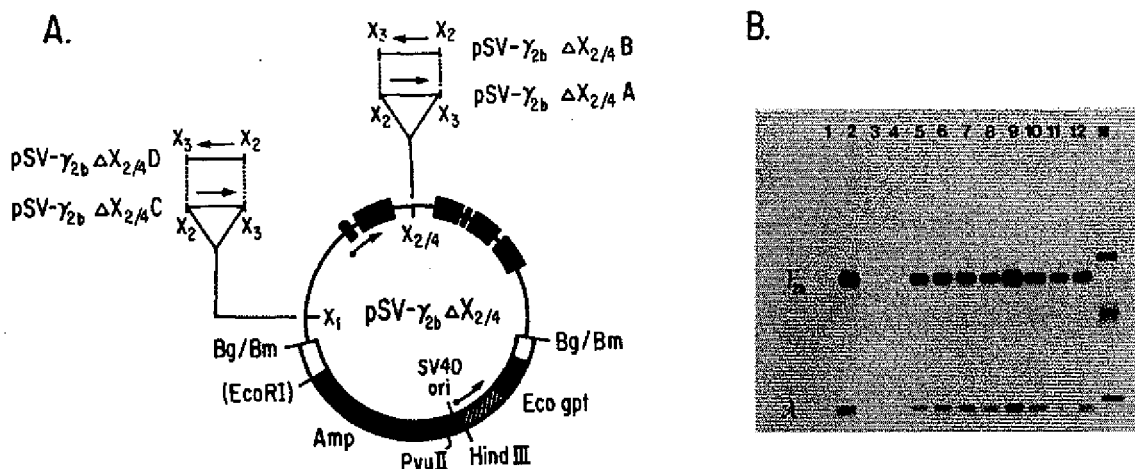


Figure 5. Enhancement of  $\gamma_{2b}$  Gene Expression by a 1 kb Intron Fragment

(A) Restriction map of plasmid pSV- $\gamma_{2b}$  $\Delta X_{2/4}$ . This plasmid was constructed from plasmid pSV- $\gamma_{2b}$ VC (shown in Figure 1) by removing two Xba I fragments from the  $\gamma_{2b}$  gene intron (from the  $X_2$  to the  $X_4$  sites in Figure 1). Derivatives of plasmid pSV- $\gamma_{2b}$  $\Delta X_{2/4}$ , labeled A through D, contain inserts of the 1 kb  $X_{2/4}$  fragment in the sites indicated and the orientation (relative to transcription) is shown with an arrow. (B) Expression of  $\gamma_{2b}$  heavy chain in cells transfected with the plasmids shown in (A). Analysis was carried out as described in Figure 2. Cell lines tested were J558L (lane 1), and those transfected with plasmid pSV- $\gamma_{2b}$ VC (lane 2), plasmid pSV2- $\gamma_{2b}$  $\Delta X_{2/4}$  (lanes 3 and 4), plasmid pSV- $\gamma_{2b}$  $\Delta X_{2/4}$ A (lanes 5 and 6), plasmid pSV- $\gamma_{2b}$  $\Delta X_{2/4}$ B (lanes 7 and 8), plasmid pSV- $\gamma_{2b}$  $\Delta X_{2/4}$ C (lanes 9 and 10), and plasmid pSV- $\gamma_{2b}$  $\Delta X_{2/4}$ D (lanes 11 and 12).

cotransfected with the herpes virus tk gene and plasmid pPL- $\gamma_{2b}$ VC (Gillies and Tonegawa, 1983). The level of  $\gamma_{2b}$  gene expression in these cells was found to be proportional to the number of transfected genes, but is at least two orders of magnitude less per gene copy than in myeloma cells. Thus it is likely that the enhancer element, described above, does not function in nonlymphoid cells.

In order to compare the levels of  $\gamma_{2b}$  gene expression in fibroblasts, with and without the immunoglobulin enhancer, we first made a deletion mutant, pPL- $\gamma_{2b}$  $\Delta X_{2/3}$ , lacking these sequences (Figure 6A). We then modified the plasmids so that a high copy number of  $\gamma_{2b}$  genes would be integrated into the transfected L cells and increase the expression of the  $\gamma_{2b}$  gene to an easily detectable level. This was done by inserting a truncated tk gene (a 2.3 kb Eco RI fragment containing only limited 5' upstream sequences) into both the wild type and mutant plasmids. Transformation to the tk<sup>+</sup> phenotype with this fragment requires the transfer of multiple plasmid copies into cells, thus another gene on the same plasmid would also be present at a high copy number in tk<sup>+</sup> transformants (our unpublished results).

Plasmids pPL- $\gamma_{2b}$ -TK and pPL- $\gamma_{2b}$  $\Delta X_{2/3}$ -TK were introduced into mouse Ltk<sup>-</sup> cells and the tk<sup>+</sup> transformants (approximately 50 individual clones) were pooled, grown in mass culture, and tested for the presence of  $\gamma_{2b}$  DNA sequences. As seen in Figure 6B, each transfected cell line contained comparable numbers of tandem, head-to-tail oligomers of either plasmid. Control experiments (not shown) indicate that individually cloned cell lines also contain the same number (about 15 copies per cell) of transfected plasmid DNA. Apparently the copy number is determined by the level of expression of the tk gene which,

in this case, has been reduced considerably by the deletion of the upstream sequences. To compensate for the low level of expression, multiple copies of the tk gene are required for tk transformation. This, then, is analogous to the results with pSV2gpt vectors described above.

We compared the expression of the normal and mutant  $\gamma_{2b}$  heavy chain genes in these cell lines by Northern gel blotting analysis of total cell RNA. As seen in Figure 6C, the steady-state level of  $\gamma_{2b}$  mRNA is not affected by the deletion of the immunoglobulin enhancer. We concluded that the low level expression of the heavy chain gene in L cells is a result of the fact that this enhancer element is functional only in lymphoid cells.

Additional experiments have been carried out to test the tissue specificity of the immunoglobulin enhancer. We constructed a derivative (pSER) of plasmid pSV2gpt lacking most of the SV40 72 bp repeat sequence (see Experimental Procedures). When this plasmid is used to transfect either mouse L cells or J558L myeloma cells, the transformation frequency (relative to that of plasmid pSV2gpt) is lowered by more than a factor of 20 (from  $2 \times 10^{-3}$  to  $10^{-4}$  in L cells and from  $3 \times 10^{-4}$  to  $10^{-5}$  in J558L myeloma cells—Table 1). When the 1 kb  $X_{2/3}$  fragment containing the immunoglobulin enhancer is inserted into the Eco RI site of plasmid pSER, the transformation frequency is restored to the level of plasmid pSV2gpt, but only in myeloma cells. There is no effect on the transformation frequency of plasmid pSER in L cells (Table 1). Thus the enhancing effect on the heterologous SV40 promoter (which controls the Ecogpt gene) is also tissue-specific.

Using this same transformation assay we tested smaller restriction fragments for enhancer activity. A 140 bp Pvu II-Dde I fragment (see Figure 1), containing some of the

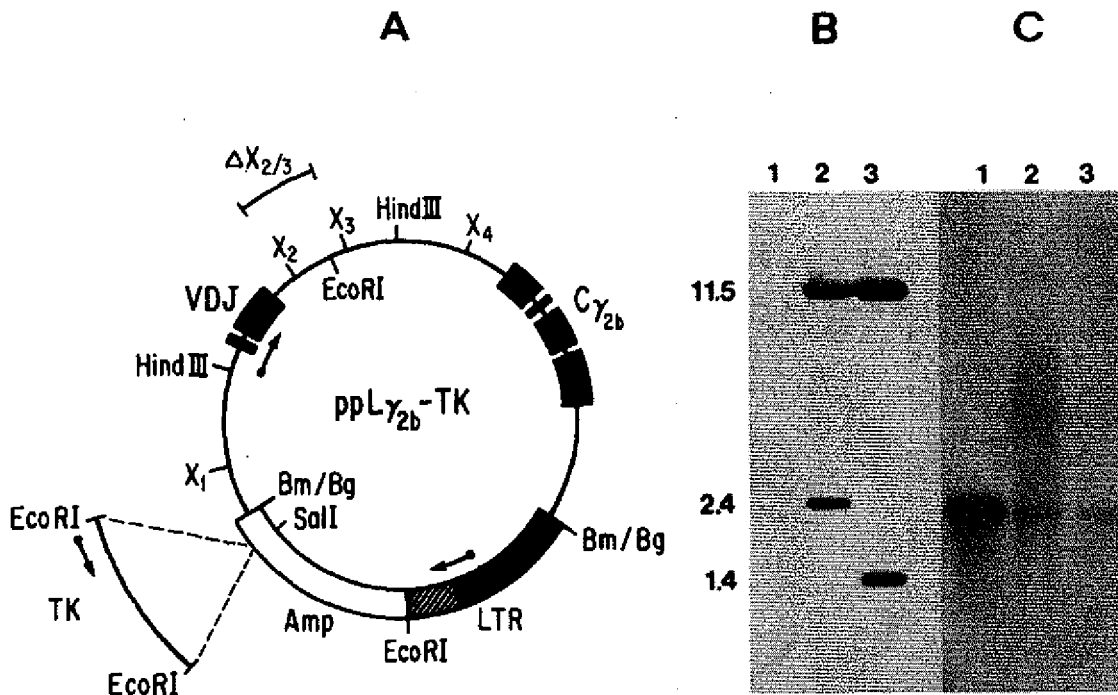


Figure 6. Expression of  $\gamma_{2b}$  mRNA in Mouse L Cells Transfected with Plasmids pPL- $\gamma_{2b}$ -tk and pPL- $\gamma_{2b}$ - $\Delta$ X<sub>2/3</sub>-tk

(A) Restriction map of the plasmids used for transfection. Plasmid pPL- $\gamma_{2b}$ -tk was constructed by inserting a 2.3 kb fragment of the herpes virus tk gene into the unique Sal I site of plasmid pPL- $\gamma_{2b}$ VC (Gillies and Tonegawa, 1983). The direction of transcription of the tk gene (arrow) is opposite that of the  $\gamma_{2b}$  gene. The sequences deleted in plasmid pPL- $\gamma_{2b}$ - $\Delta$ X<sub>2/3</sub>-tk are indicated. (B) Southern gel blotting analysis of DNA from L TK<sup>-</sup> cells (lane 1), and cells transfected with plasmid pPL- $\gamma_{2b}$ -tk (lane 2), or plasmid pPL- $\gamma_{2b}$ - $\Delta$ X<sub>2/3</sub>-tk (lane 3). DNA was digested with Hind III and hybridized to nick-translated  $\gamma_{2b}$  DNA (the 9 kb Bgl II fragment used for plasmid construction). (C) Northern gel blotting analysis of total cell RNA from MOPC 141 myeloma cells (lane 1), and cells transfected with plasmid pPL- $\gamma_{2b}$ -tk (lane 2), or plasmid pPL- $\gamma_{2b}$ - $\Delta$ X<sub>2/3</sub>-tk (lane 3). Nick-translated C $\gamma_{2b}$  probe (Gillies and Tonegawa, 1983) was used for hybridization.

sequences deleted in plasmid pSV- $\gamma_{2b}$ 3'RA2, was found to increase the transformation frequency of plasmid pSER by 20-fold in J558L cells but not in mouse L cells (Table 1). Thus we have localized the immunoglobulin enhancer sequence to this portion of the X<sub>2/3</sub> fragment.

#### DNA Sequences in the Heavy Chain Gene Intron Resemble Viral Enhancers

Weiherr et al. (1983) have suggested that the sequence 5' GTGG<sup>AAA</sup><sub>TTT</sub>G3' (where <sup>A</sup><sub>T</sub> means either A or T appears at that position) represents a crucial core element common to all of the known viral enhancers. Sequence analysis of the X<sub>2/3</sub> fragment (the 1 kb fragment shown to have enhancer activity—Figure 5) shows that such a sequence is located in the region that is deleted in plasmid pSV- $\gamma_{2b}$ 3'RA2 (but not in the 3'RA1 mutant) and is present in the 140 bp Pvu II-Dde I fragment. In fact, the sequence 5'GTGGTTT(T)GAA-3' is present as a closely spaced repeat (Figure 7), oriented in the direction of transcription. The first eight nucleotides of this sequence are also found upstream of the tandem repeat, but oriented in the opposite direction.

Figure 8 shows a comparison of several viral sequences shown to have enhancer activity and the repeat sequences

Table 1. Transformation Frequency of pSV2gpt and Derivative Plasmids in J558L Myeloma Cells and L Cells

Plasmid	Cell Type	
	J558L	L Cell
pSV2gpt	$3 \times 10^{-4}$	$2 \times 10^{-3}$
pSER	$8 \times 10^{-6}$	$1 \times 10^{-4}$
pSER-X <sub>2/3</sub>	$4 \times 10^{-4}$	$1 \times 10^{-4}$
pSER-X <sub>2/3</sub> (140)	$2 \times 10^{-4}$	$9 \times 10^{-6}$

Cells were transfected by protoplast fusion and plated at  $10^4$  cells per well and  $2 \times 10^3$  cells per well (J558L) or at  $10^4$  and  $10^5$  cells per 100 mm dish (L cells). Selective medium containing mycophenolic acid (6  $\mu$ g/ml for J558L or 25  $\mu$ g/ml for L cells) was added at 48 hr and colonies were counted at 10 days (J558L) or at 14 days (L cells). Derivatives of plasmid pSER were constructed by inserting (blunt-end ligating) either the 1 kb X<sub>2/3</sub> fragment or a 140 bp Pvu II-Dde I [X<sub>2/3</sub>(140)] fragment (see Figure 1) into the Eco RI site.

in the  $\gamma_{2b}$  gene intron. Sequences contained in the Moloney sarcoma virus (MSV) 73 bp repeat sequence appear to be most similar to the immunoglobulin sequence, especially on the 5' side of the first "core" repeat. The "core" sequence of polyoma virus was most similar to the second "core" repeat, as both contain an additional T residue.

Also shown are two sequences, present in the immu-

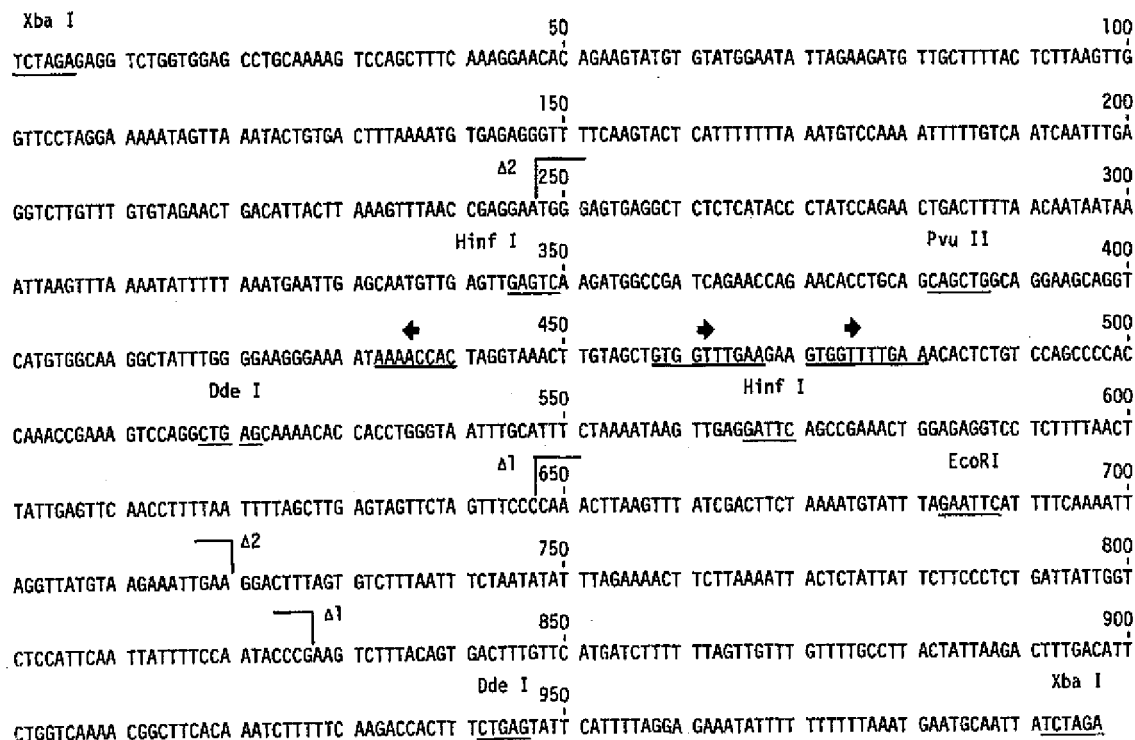


Figure 7. Nucleotide Sequence of the 1 kb X<sub>ba</sub> Fragment

DNA sequencing was carried out according to standard procedures (Maxam and Gilbert, 1980). The sequences deleted in plasmids pSV-γ<sub>2b</sub>3'RA1 and pSV-γ<sub>2b</sub>3'RA2 are indicated. The underlined sequences are those similar to the "core" elements common to most viral enhancers (Weiher et al., 1983). Arrows indicate the orientation of the immunoglobulin "core" elements relative to the direction of γ<sub>2b</sub> mRNA transcription.

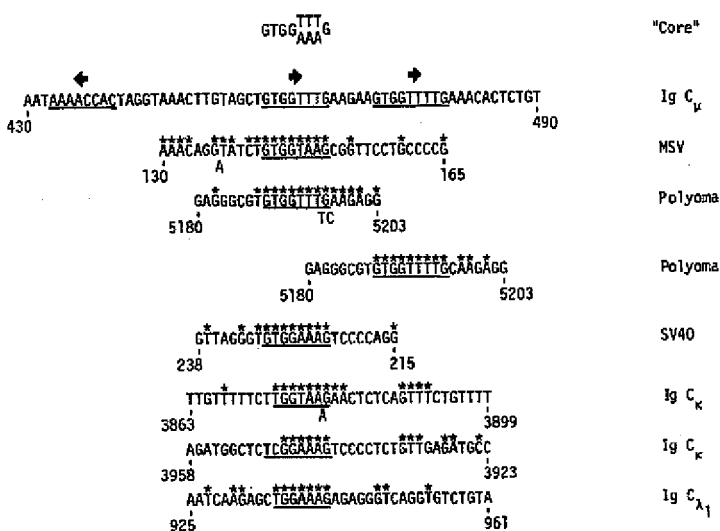


Figure 8. A Comparison of Sequences in the 1 kb X<sub>ba</sub> Fragment with Those of Viral Enhancer Elements

Sequences are aligned at the putative "core" (underlined) sequences (Weiher et al., 1983). The residues which match those in the immunoglobulin heavy chain sequence are indicated by an asterisk. These include either an A or T residue in positions 5-7 of the "core" sequence. In some cases a residue has been displaced to maximize the homology. Numberings of the sequences are according to Van Beveren et al., 1981 (MSV); Griffin et al., 1980 (Polyoma); Buchman et al., 1980 (SV40); Max et al., 1981 (Ig C<sub>κ</sub>); Bernard and Tonegawa, unpublished data (Ig C<sub>λ1</sub>).

noglobulin light chain gene intron, that resemble an enhancer element. The existence of such a sequence near the C<sub>κ</sub> gene segment is rendered plausible by its proximity to a DNAase I hypersensitive site (Parslow and Granner, 1982). In addition, studies using transfected genes suggest

that deletions in this region reduce transcription from the V promoter (V. O. and S. M., unpublished data). A similar core-like sequence is also present near the constant portion of the λ<sub>1</sub> light chain gene, but there is yet no evidence showing that this is part of an enhancer element.

## Discussion

### Evidence for an Enhancer Element in the Intron of a Heavy Chain Immunoglobulin Gene

Sequences contained in the major intron between the functionally rearranged VDJ and C exons of a heavy chain immunoglobulin gene were shown to be essential for its high level expression in transfected myeloma cells. Although the deletion of these sequences decreases the level of expression in transfected cells 20 times (Figure 2), the actual reduction is probably about 400 times per gene copy. We have tested whether these sequences are analogous to viral enhancer elements. In addition to increasing the level of transcription from homologous promoters, viral enhancers also increase transcription from many heterologous, viral or nonviral promoters. This enhancing activity is independent of the orientation of the enhancer element, relative to the direction of transcription, and is independent of its position as long as the distance between the enhancer and promoter is within several kilobases (Banerji et al., 1981; Moreau et al., 1981; Wasylyk et al., 1983).

These properties also apply to the sequences contained in the heavy chain immunoglobulin gene. Enhancement of the heterologous SV40 promoter occurs when the intact  $\gamma_{2b}$  heavy chain gene is present in plasmid pSV2gpt and a low copy number of the recombinant plasmid is sufficient for gpt transformation. When the  $\gamma_{2b}$  gene intron sequences are removed, the plasmid copy number increases dramatically to compensate for the decreased expression of the gpt gene from the SV40 promoter (Figure 4).

We also found that DNA fragments from the  $\gamma_{2b}$  gene intron can substitute for the SV40 enhancer in plasmid pSV2gpt (Table 1). This transformation assay is based on the ability of the DNA fragments, located more than 2 kb away from the SV40 promoter, to enhance the transcription of the gpt gene and thereby increase the transformation

frequency. Using this method we have shown that most of the enhancing activity can be localized to a 140 bp fragment. The nucleotide sequence in this region contains a repeat sequence which closely resembles the "core" nucleotides found in most viral enhancers (Figure 8).

Finally, we demonstrated that this intron sequence maintains its ability to stimulate transcription of the heavy chain gene when it is moved outside of the  $\gamma_{2b}$  transcription unit (5' of the mRNA start site) and when its orientation is reversed (Figure 5). These results show that the immunoglobulin intron sequence is an enhancer element and has properties in common with those of viral origin, even though the latter are generally located on the 5' side of their cognate transcriptional promoters.

### The Role of Transcriptional Enhancement in the Regulation of Immunoglobulin Gene Expression

The creation of active immunoglobulin genes through somatic recombination has been studied in detail (reviewed by Tonegawa, 1983), but the mechanism by which this activation is brought about has been a major problem of molecular immunology. The observation that the  $C_\mu$  and  $C_\gamma$  gene segments are transcriptionally active in lymphoid cells, prior to V-J or V-D-J joining (Kemp et al., 1980; Van Ness et al., 1982), provided the first evidence that sequences downstream of the V gene segment promoter might affect the transcription of the functionally rearranged gene.

The mechanism of this activation can now be explained, at least for the heavy chain gene, by our identification of an enhancer element between the  $J_H$  and  $C_{\gamma_{2b}}$  gene segments of a functionally rearranged gene. This site corresponds to the  $J_H$ - $C_\mu$  region of germ-line DNA (see Figure 9). Following VDJ-joining, which occurs before B cells encounter antigens, this enhancer (which would now be part of the major intron of the functionally rearranged  $\mu$

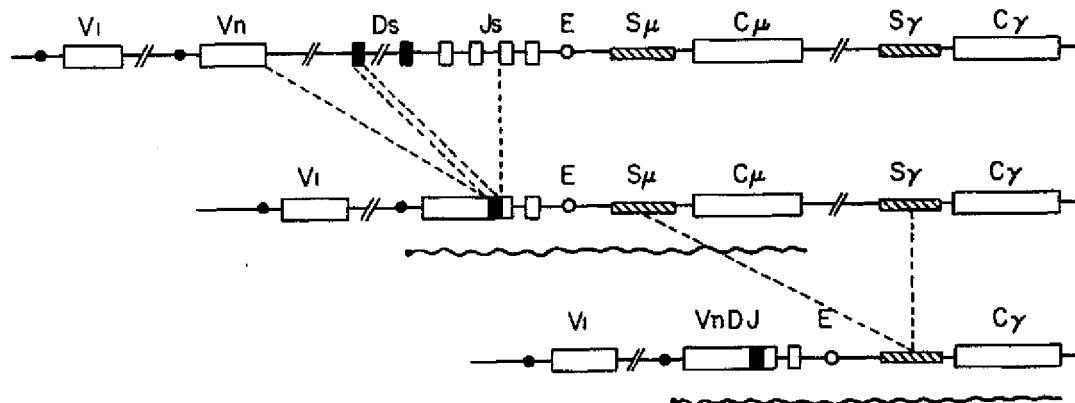


Figure 9. Schematic Diagram Showing Arrangements of Various Ig Heavy Chain Gene Segments and the Position of the Enhancer

Top, middle, and bottom arrangements correspond to germ-line DNA;  $\mu$  chain-positive, pre-switch B cell DNA; and  $\gamma$  chain-positive, post-switch plasma cell DNA, respectively. The enhancer element (C) is located between the  $J_H$  segments and  $C_\mu$  segments in the germ-line DNA and becomes part of the major intron in the active  $\mu$  chain gene upon V-D-J joining. The same enhancer element is retained in the major intron of the active  $\gamma$  chain gene, which is created by a switch recombination from the  $\mu$  gene.  $S_\mu$  and  $S_\gamma$  refer to the regions in which switch recombinations occur. The wavy lines and the large filled circles (●) represent the primary transcripts and the promoters, respectively. The small filled circles (•) represent the 5' caps of the RNA molecules.

chain gene) activates the promoter of the rearranged V gene segment. In this way only a single V gene segment (out of several hundred) would be transcriptionally active, and only after functional rearrangement had occurred.

Subsequent to the encounter with antigens and stimulation by T cells, a second type of rearrangement (switch recombination) occurs in heavy chain genes and results in the replacement of the C<sub>μ</sub> coding sequence (Figure 9) with those of the other heavy chain classes and subclasses (Maki et al., 1980; Kataoka et al., 1980; Sakano et al., 1980; Davis et al., 1980). In order to function after class switching, the immunoglobulin enhancer would have to be located upstream of the switch region, otherwise it would be deleted along with the C<sub>μ</sub> coding sequence. This is in fact the case since its location is more than 1 kb upstream of any known switch sites within the S<sub>μ</sub> region. Thus as we have shown for the MOPC 141 γ<sub>2b</sub> gene, it is likely that the same enhancer is used for the expression of all heavy chain classes following switch recombination, although it is possible that additional regulatory elements may be associated with the individual C region gene segments.

#### Evidence for Other Cellular Enhancer Elements

The data presented in this report represents the first clear demonstration of an enhancer element being associated with a defined cellular gene. The possibility that enhancers are present in cellular DNA has already been suggested by others. For example, Conrad and Botchan (1982) isolated human DNA sequences which hybridized to the region of SV40 DNA spanning the origin of replication. One of these sequences was found to enhance the efficiency of tk transformation in an orientation-independent manner and thus resembles the viral enhancer elements. Furthermore, this DNA sequence cross-hybridized with many sequences in human DNA, suggesting that a family of such elements exists.

Rosenthal and Khoury (personal communication) have likewise isolated a human DNA sequence by virtue of its cross-hybridization with a portion of BK virus DNA. In this case, however, the sequence appears to be unique in the human genome even though it contains repeating 21 bp elements. These repeat elements show some homology to the BK virus enhancer region but appear to be about 8 times less active when tested for enhancing activity in the CAT assay of Gorman et al. (1982).

Thus it is likely that enhancer elements might serve as a general mechanism for gene regulation in eucaryotes. The association of such elements with specific genes is currently being studied in several systems. In addition to our demonstration of an enhancer element in the heavy chain immunoglobulin gene, two of us (V. O. and S. M.) have found that sequences near the C<sub>κ</sub> gene segment are essential for the high level expression of κ chains in transfected myeloma cells. In this case, however, the functional similarity of this sequence element to the viral enhancers is less clear.

The use of enhancer elements as regulators of gene

expression may not be confined to higher eucaryotic systems. There is evidence (L. Guarente, personal communication) that the yeast iso-1-cytochrome c gene is activated by heme and that sequences upstream of the gene are essential for this effect. Furthermore, the inversion of this activator sequence did not affect the inducibility of expression. This strongly suggests that this region is not simply a component of the transcriptional promoter.

#### Tissue-specific Enhancer Elements and Their Possible Role in Cell Differentiation

The most interesting property of the immunoglobulin enhancer is its tissue specificity. The MSV and SV40 viral enhancers have been shown to have a certain degree of host cell specificity (Laimins et al., 1982). This effect may also be explained in terms of tissue specificity because the two cell types used for the comparison were derived from different tissues. The immunoglobulin enhancer, on the other hand, functions at a high level in a lymphoid (myeloma) cell type but not at all in another cell type (fibroblast) of the same species. It is likely that this specificity is the result of a factor (or factors), present only in lymphoid cells, which regulates immunoglobulin expression during B cell ontogeny.

At early stages of B cell development, μ heavy chains are expressed at a low level (Levitt and Cooper, 1980). After the B cell encounters antigen and interacts with regulatory T cells, terminally differentiated plasma cells appear and produce very high levels of immunoglobulin (Schibler et al., 1978). The quantitative differences in the level of immunoglobulin gene expression at different stages of B cell development suggest that the enhancer function may be stage-specific. It is also possible that multiple regulatory elements are contained within this enhancer region and that increased levels of expression result from the combined effect of individual enhancers.

Another possible example of a tissue-specific enhancer element has been described in the polyoma virus system (Katinka et al., 1980; Fujimura et al., 1981; Fujimura and Linney, 1982). It was shown that polyoma mutants that acquire the ability to replicate in the otherwise refractory F9 embryonal carcinoma cells contain point mutations and, in some cases, tandem duplications near the region of polyoma DNA which was shown (de Villiers et al., 1981) to have enhancer activity. This result suggests that certain DNA sequences are recognized as an enhancer in cells permissive for polyoma virus but that sequences located at an adjacent site are recognized (after being mutated) in embryonal cells. Thus it appears that polyoma, like immunoglobulin genes, may contain multiple regulatory elements within their enhancer regions.

The mechanism of tissue-specific enhancer is not known and this simply reflects our present ignorance of the mechanism of enhancers in general. Clearly though, the sequence differences and similarities between the various enhancers strongly suggest that specific regulatory proteins recognize these sites. In fact, the glucocorticoid

receptor protein, which binds to the promoter region of mouse mammary tumor virus (MMTV), may be an example of an enhancer binding protein (K. Yamamoto, personal communication). A sequence upstream of the MMTV promoter, essential for hormone responsiveness, was shown to enhance the herpes *tk* gene in an orientation-independent manner and to bind the hormone receptor.

It is tempting to speculate that the presence or absence of such enhancer binding proteins determines whether or not an enhancer functions in a given cell type. Furthermore, a particular enhancer binding protein might recognize many different but related sequences to a greater or lesser extent (or bind with different affinities). In this way it would be possible to activate many individual genes and to express them at different levels. Alternatively, the level of expression of a gene that is controlled by an enhancer may be determined by the distance between this element and the promoter site. It seems likely that some or all of these mechanisms of gene regulation function during the process of cellular differentiation. In this way the expression of multiple genes could be controlled (coordinately expressed) by a relatively small number of regulatory proteins.

### Enhancers as Activators of Cellular Oncogenes

One striking case for the role of enhancers in tumorigenesis was provided by studies of avian leukosis virus (ALV) induced chicken B cell lymphomas. In such lymphomas, ALV DNA was found to be integrated adjacent to the *c-myc* gene (Payne et al., 1981), the cellular counterpart of the transforming sequences from the MC29 group of defective retroviruses (Sheiness and Bishop, 1979). Although it was first thought that *c-myc* was activated by a promoter-insertion mechanism (Hayward et al., 1981), it was later shown that ALV insertions could occur in the opposite orientation or downstream of the *c-myc* gene (Payne et al., 1982). Thus the ALV enhancer element was responsible for the increased level of *c-myc* expression and, presumably, for oncogenic transformation.

The role of cellular enhancer elements in the activation of oncogenes has also been suggested by recent findings (reviewed by Klein, 1983). Many murine and human tumors of lymphoid origin have been shown to contain chromosomal translocations in which an oncogene (*c-myc*) has been rearranged to an immunoglobulin C region gene segment. The majority of rearrangements in human Burkitt lymphomas were found to occur at the  $C_{\mu}$  region while those in mouse plasmacytomas occur at the  $C_{\alpha}$  region. While the results we have described could account for the activation of *c-myc* in some human  $C_{\mu}$  rearrangements (by analogy to the murine  $C_{\mu}$  enhancer), the results in the murine system are somewhat unclear. It has not been ruled out, however, that the murine  $C_{\alpha}$  gene segment contains an additional enhancer element.

We are currently investigating the activation of *c-myc* by sequences contained near the *c-myc*- $C_{\mu}$  junction in human DNA. It will be interesting to compare the se-

quences in this region with those that we showed to contain enhancer activity. Sequences that have been conserved through evolution may also help to identify the critical components of this regulatory element.

### Experimental Procedures

#### Cell Culture and Transfection

The myeloma cell line, J558L, is a heavy chain loss variant of J558 and synthesizes  $\lambda$  light chains (Oi et al., 1983). Cells were grown in Dulbecco's modified Eagle's medium (MEM) containing 10% fetal calf serum. J558L cells were transfected by a modification of the protoplast fusion technique (Sandri-Goldin et al., 1981). Approximately  $2 \times 10^6$  cells (grown to a density of  $4$  to  $6 \times 10^5$  cells/ml) were washed once with serum-free MEM, collected by centrifugation (5 min. at 500 g), and suspended by gentle pipetting in the protoplast suspension (approximately  $2 \times 10^6$  protoplasts in 4 ml). The cell-protoplast suspension was transferred to a 60 mm dish and centrifuged at 1500 g for 7 min. After gentle aspiration of the supernatant, 1.5 ml of 50% PEG-1500 (in serum-free MEM and prewarmed to 37°C) was added and the dish was spun at 500 g until 90 sec had elapsed from the time of PEG addition. Cells were resuspended by gently pipetting in two 5 ml washes of prewarmed, serum-free MEM which were added to 15 ml of MEM in a 50 ml centrifuge tube. Following centrifugation at 500 g for 5 min, cells were resuspended in growth medium containing kanamycin (100  $\mu$ g/ml) and plated in 96-well dishes at two densities:  $1 \times 10^4$  cells per well and  $2 \times 10^5$  cells per well. After 48 hr selective medium (Oi et al., 1983) was added.

#### Plasmid Constructions

Plasmid pSV- $\gamma_{2b}$ VC, containing the  $\gamma_{2b}$  gene from myeloma MOPC 141, was constructed by inserting a 9 kb Bgl II fragment (Gillies and Tonegawa, 1983) from phage clone M141-p21 (Sakano et al., 1980) into the unique Bam HI site of plasmid pSV2gpt(Ri). This latter plasmid was constructed by mutating the Eco RI site of plasmid pSV2gpt (Mulligan and Berg, 1980). The transcription orientation of the  $\gamma_{2b}$  gene is opposite that of the gpt gene (Figure 1).

Plasmids pSV- $\gamma_{2b}$ 3'RA1 and pSV- $\gamma_{2b}$ 3'RA2 were constructed by digesting Eco RI-cut pSV- $\gamma_{2b}$ VC DNA with exonuclease Bal 31 (1 U/ $\mu$ g of DNA) at 23°C for 2 or 4 min and recircularizing the products with T4 DNA ligase. The extent of the deletions were determined by restriction analysis and DNA sequencing.

Plasmid pSV- $\gamma_{2b}$  $\Delta X_{24}$  was constructed by first digesting plasmid pSV- $\gamma_{2b}$ VC DNA with Bgl II and then partially digesting with Xba I. The 6.5 kb partial digestion product extending from the unique Bgl II site to the Xba I site ( $X_2$ ) on the 3' side of the VDJ exon (clockwise on the map in Figure 1) and the 5.1 kb complete digestion product extending from the Bgl II site, counterclockwise to the Xba I site ( $X_4$ ) on the 5' side of the  $C_{\gamma_{2b}}$  coding region, were gel purified and ligated. The resulting plasmid, pSV- $\gamma_{2b}$  $\Delta X_{24}$ , was used for the experiment shown in Figure 5. Derivatives of this plasmid (A-D) were constructed by partially digesting with Xba I, treating the DNA with calf intestine alkaline phosphatase, purifying linear full-length DNA, and ligating the products with the 1 kb Xba I fragment extending from the  $X_2$  to  $X_4$  sites ( $X_{24}$  fragment in Figure 1). The site of insertion and the orientation of the  $X_{24}$  fragment were determined by restriction analysis.

Plasmid pSER was constructed by digesting plasmid pSV2gpt DNA with Sph I and Pvu II and removing the 3' protruding bases with T4 DNA polymerase (O'Farrell, 1981). The two blunt ends were then ligated to produce a selectable plasmid vector which no longer contains the SV40 enhancer sequence.

#### Analysis of Transfected Cells

Approximately 10 days after transfection, the cells contained in a single well (from 5 to 10 independent clones) were harvested and grown in mass culture for analysis of protein synthesis and the steady-state level of  $\gamma_{2b}$  mRNA (Gillies and Tonegawa, 1983). Four such pools were analyzed for each plasmid tested as well as subclones obtained by limiting dilution.

Protein synthesis was measured by labeling  $5 \times 10^6$  cells for 1 hr with  $^{35}$ S-methionine (50  $\mu$ Ci/ml) and analyzing immunoprecipitated cell extracts as described (Gillies and Tonegawa, 1983). Secretion of immunoglobulin

was measured by labeling approximately  $2 \times 10^4$  cells for 16 hr in 50  $\mu$ l of normal growth medium containing  $^{35}$ S-methionine (25  $\mu$ Ci/ml). Immunoglobulin was then immunoprecipitated from culture supernatants.

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# Cell-Type Specificity of Immunoglobulin Gene Expression is Regulated by at Least Three DNA Sequence Elements

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## Summary

The regulation of cell-type specificity of immunoglobulin (Ig)  $\mu$  heavy chain (H) gene expression was examined by introducing various hybrid genes containing Ig gene sequences combined with portions of a tissue-nonspecific transcription unit into lymphoid and nonlymphoid cells. Replacing the lymphocyte-specific IgH enhancer with a viral enhancer did not affect tissue specificity of  $\mu$  Ig gene expression. We identified two new regulatory regions that provide transcriptional tissue specificity. First, the  $V_H$  promoter region between position -154 and +57 was shown to direct lymphocyte-specific transcription of a bacterial *gpt* gene, even in the presence of a viral enhancer. Second,  $\mu$  intragenic sequences, lacking the IgH enhancer, were found to regulate the level of accumulated Ig transcripts in a tissue-specific fashion. These results demonstrate that tissue specificity of Ig gene expression is not solely regulated by the enhancer but that the promoter, and as yet undefined intragenic sequences, contain lymphoid-specific regulatory information.

## Introduction

Immunoglobulin (Ig) genes are expressed specifically in cells of the B lymphocyte lineage. Very early in B cell differentiation the heavy chain (H) locus becomes transcriptionally active, and the variable (V) gene segment is assembled from its various components by DNA rearrangement (Tonegawa, 1983). DNA rearrangement is accomplished in two steps, D-to-J<sub>H</sub> and V<sub>H</sub>-to-DJ<sub>H</sub> joining, with the first rearrangement sometimes occurring in T cells (Kurosawa, 1981; Alt et al., 1984). Somatic recombination is not required for the transcriptional activation of the heavy chain gene because even incompletely rearranged or unrearranged genes are transcribed from pseudopromoters 5' to the constant (C) region (Kemp et al., 1980; Alt et al., 1982; Nelson et al., 1983). In completely rearranged genes the promoter 5' to the V gene segment is used (Clarke et al., 1982). Transcription of the heavy chain gene is tissue-specific and restricted to B and T lymphocytes, implying that lymphoid cells contain specific *trans*-acting factors required for heavy chain gene activation. Because complete V<sub>H</sub>DJ<sub>H</sub> joining is restricted to B cells, only these cells can synthesize functional heavy chains.

Within the B lymphoid cell lineage, expression of the heavy chain gene is cell-type-specific. In early stage cells,

termed pre-B cells, transcriptional activation and rearrangement of the heavy chain gene occurs (Maki et al., 1980; Alt et al., 1981). In mature B cells, light chain genes are expressed, and surface immunoglobulins are synthesized (Alt et al., 1980; Rogers et al., 1980). In both cell types, the level of accumulated Ig mRNA is low (Perry and Kelley, 1979). When B cells encounter antigen, the cells mature into terminally differentiated plasma cells, and the amount of accumulated Ig mRNA is increased 30- to 100-fold (Perry and Kelley, 1979). Most of this dramatic increase in Ig gene transcript accumulation is due to post-transcriptional regulation because the rate of transcription in plasma cells is accelerated only 2- to 5-fold over the rate in B cells (Mather et al., 1984).

Gene transfer experiments, in which cloned rearranged wild-type or in vitro mutated Ig genes have been introduced into terminally differentiated lymphoid and nonlymphoid cells, have pointed to certain mechanisms of Ig gene regulation. Two regulatory elements have been described that are crucial for the expression of Ig genes in myeloma cells, the tumor cell counterpart of plasma cells. One element, residing in the intron between the variable and constant regions of both heavy and  $\kappa$  light chain genes, acts as a transcriptional enhancer (Banerji et al., 1983; Gillies et al., 1983; Queen and Baltimore, 1983; Picard and Schaffner, 1984; Bergman et al., 1984; Potter et al., 1984). Because the heavy chain enhancer is inoperative in nonlymphoid cells, it has been suggested that this regulatory element controls the tissue-specific expression of Ig genes (Banerji et al., 1983; Gillies et al., 1983). Another sequence element located about 30 bp upstream of the TATA box was found to be required for  $\kappa$  gene transcription in myeloma cells (Falkner and Zachau, 1984; Bergman et al., 1984).

To investigate which sequence elements are regulating tissue specificity and modulation of Ig gene transcription during B cell differentiation, we have dissected the heavy chain transcription unit into enhancer, promoter, and intragenic sequences and tested these elements individually for their contribution to regulation of Ig gene expression. We show here that each of the three regulatory elements can confer tissue specificity to heterologous transcription units. The heavy chain enhancer is required for Ig gene transcription, but it can be replaced with a viral enhancer without affecting the tissue specificity of gene expression. The  $V_H$  promoter region and the  $\mu$  intragenic sequences also control the differential Ig gene expression in lymphoid versus nonlymphoid cells.

## Results

To study the expression of Ig heavy chain genes in cultured mouse cells, a functionally rearranged  $\mu$  gene containing a  $V_H$  gene segment isolated from the hybridoma 17.2.25 (Loh et al., 1983; Grosschedl et al., 1984) was inserted into a short-term expression vector (Figure 1A). The vector contained the early region of polyoma virus to in-

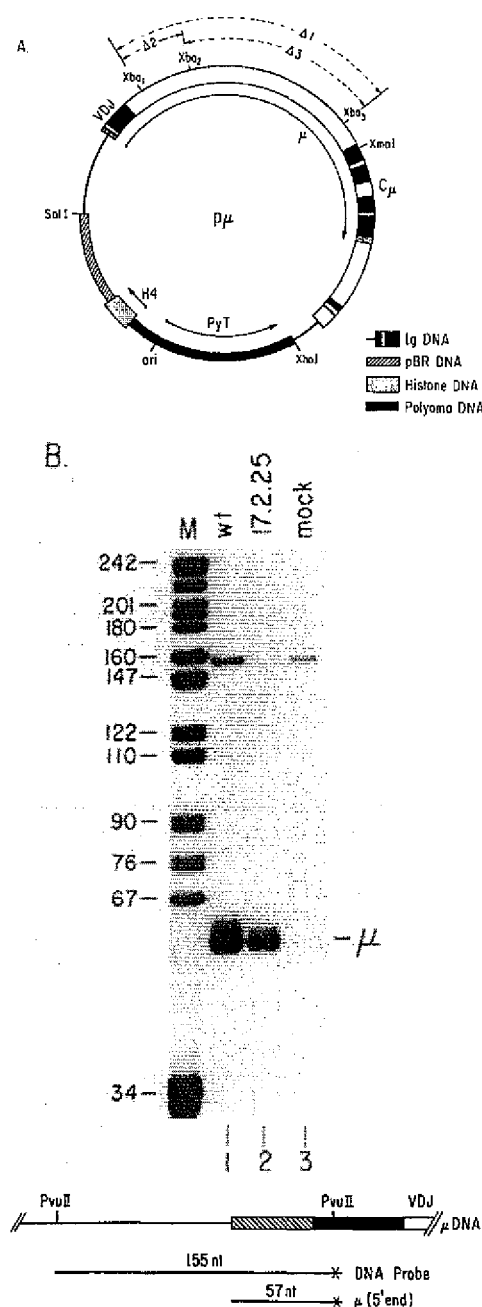


Figure 1. Structure and Expression of the Plasmid p $\mu$  in Myeloma MPC11 Cells

(A) Map of the plasmid DNA. The  $\mu$  gene with its rearranged V<sub>H</sub> gene segment isolated from the hybridoma 17.2.25 is represented by filled bars (exons), open bars (introns), and hatched bars (noncoding sequences); the 5' and 3' flanking regions of the  $\mu$  gene are shown as lines. The polyoma virus (Py) early region is depicted as a narrowed filled bar. The Py early region includes the origin of replication (ori) and DNA sequences encoding T antigens. Vector pBR322 sequences and H4 gene sequences are drawn hatched and dotted, respectively. The transcriptional polarities of the genes are indicated by arrows. The structure of deletions in the V<sub>H</sub>DJ $\mu$ /C $\mu$  intron is shown. The deleted DNA sequences are indicated by dashed lines extending between Xba I cleavage sites.

(B) S1 nuclease mapping of specific V<sub>H</sub> 17.2.25 RNA 5' ends. Total RNA (20  $\mu$ g) from myeloma MPC11 cells transfected with p $\mu$  wild-type DNA (lane 1), mock-transfected cells (lane 2), or total 17.2.25 hybridoma RNA (0.2  $\mu$ g) (lane 3) were hybridized to the 5' end-labeled DNA probe shown (bottom) and digested with S1 nuclease as described in Experimental Procedures. The protected fragments were separated by electrophoresis and revealed by autoradiography. Lane M, <sup>32</sup>P labeled Hpa II-digested pBR322 DNA as size marker. The sizes are indicated in base pairs. The arrow indicates the 57 nucleotide major protected fragment. (Bottom) the structure of the 5' end of the  $\mu$  gene is shown. The leader coding sequences are drawn as a filled bar; intron sequences are shown as an open bar and 5' noncoding sequences as a hatched bar. The structures and sizes of the single-stranded DNA probe and the protected DNA fragment are shown.

(C) Analysis of H4 transcripts. Total RNA (5  $\mu$ g) from MPC11 cells transfected with p $\mu$  DNA (lane 2) or mock-transfected cells (lane 1) was analyzed for the presence of H4 histone transcripts by S1 nuclease mapping. The position of the 99 nucleotide fragment protected by transcripts of the H4 reference gene is indicated as H4<sup>ref</sup>. Two other fragments of 175 and 145 nucleotides mapped to the initiation site of transcription and to the ATG initiation codon of the wild-type H4 gene. The 175 nucleotide protected fragment corresponded to transcripts from the endogenous H4 gene (H4<sup>wt</sup>) and the 145 nucleotide fragment corresponded to RNA from endogenous H4 variant genes (H4<sup>v</sup>) that contain sequence homology to the coding region of the DNA probe. The structure of the H4<sup>wt</sup> and H4<sup>v</sup> genes is shown at the bottom. The coding regions of the wild-type and the mutant H4 genes are depicted as filled bars. The deletion in the mutated reference gene is indicated by dashed lines. The DNA probe used for S1 nuclease mapping consisted of a 265 nucleotide Hpa II fragment that had been isolated from the wild-type H4 gene. The structure and sizes of the protected DNA fragments are shown.

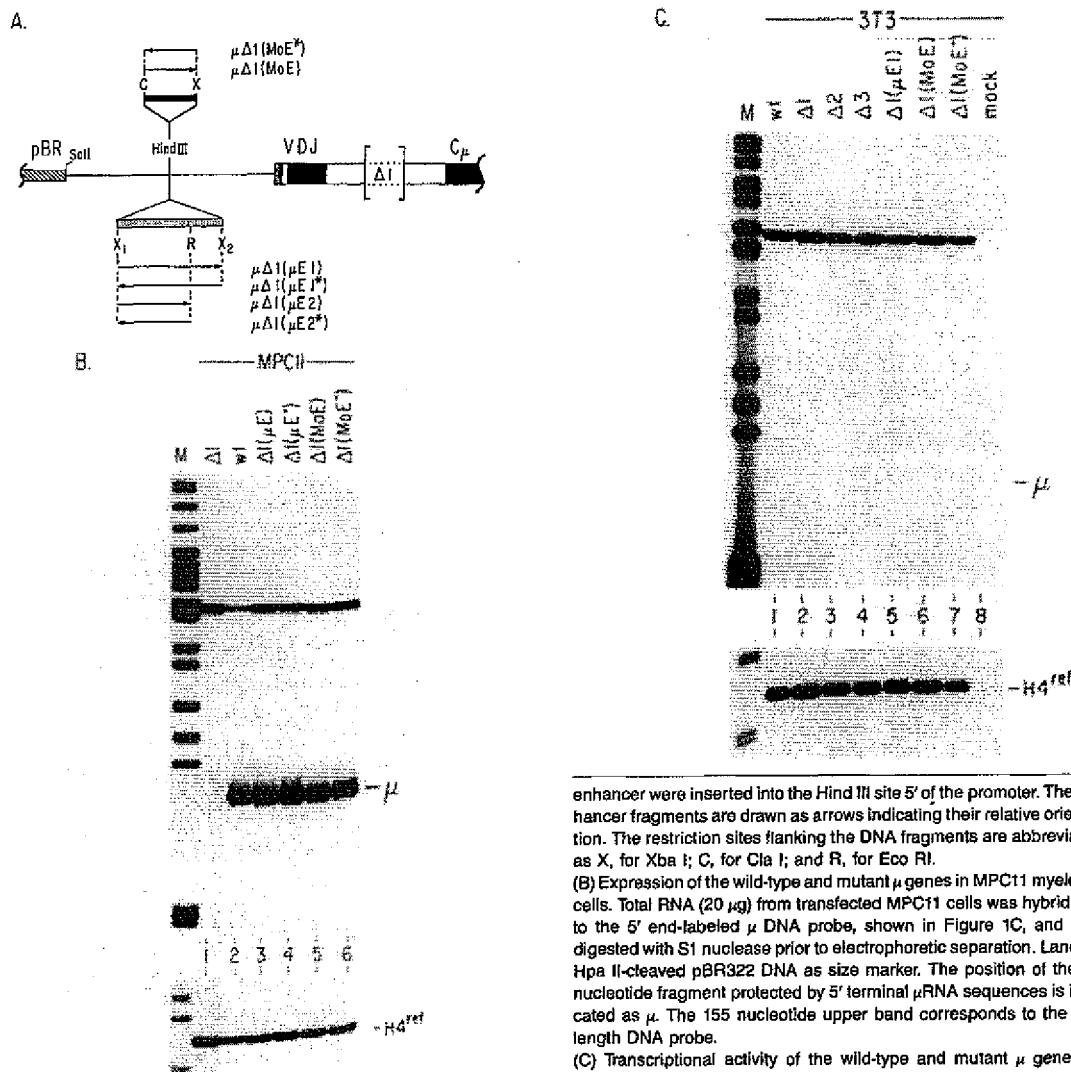


Figure 2. Expression of Wild-Type and Mutant  $\mu$  Genes in Myeloma MPC11 Cells and NIH/3T3 Fibroblasts.

(A) Structure of enhancer insertion mutations. The  $\mu\Delta 1$  gene, which is lacking all DNA sequences between the Xba I<sub>1</sub> and Xba I<sub>2</sub> sites in the J $\mu$ /C $\mu$  intron including the IgH enhancer (Figure 1A), is shown. Various DNA fragments containing either the Ig enhancer or the M-MuLV (Mo)

crease the copy number of the linked genes and thus the amount of accumulated RNA in the transfected cells (Queen and Baltimore, 1983). Control experiments with plasmids lacking the polyoma virus DNA segment, as well as the total absence of transcription when Ig regulatory sequences were deleted, suggested that the polyoma DNA segment did not perturb the regulation and expression of the  $\mu$  gene in our construct. To control for the effects of sequence alterations in the  $\mu$  gene, we included in the construct a mouse H4 histone gene, which has a small transcription unit and no obvious tissue- or cell-type specificity (Seller-Tynns and Birnstiel, 1981).

The complete DNA construct, termed p $\mu$  (Figure 1A),

enhancer were inserted into the Hind III site 5' of the promoter. The enhancer fragments are drawn as arrows indicating their relative orientation. The restriction sites flanking the DNA fragments are abbreviated as X, for Xba I; C, for Cla I; and R, for Eco RI.

(B) Expression of the wild-type and mutant  $\mu$  genes in MPC11 myeloma cells. Total RNA (20  $\mu$ g) from transfected MPC11 cells was hybridized to the 5' end-labeled  $\mu$  DNA probe, shown in Figure 1C, and was digested with S1 nuclease prior to electrophoretic separation. Lane M, Hpa II-cleaved pBR322 DNA as size marker. The position of the 57 nucleotide fragment protected by 5' terminal  $\mu$ RNA sequences is indicated as  $\mu$ . The 155 nucleotide upper band corresponds to the full-length DNA probe.

(C) Transcriptional activity of the wild-type and mutant  $\mu$  genes in NIH/3T3 fibroblasts. Total RNA from NIH/3T3 cells transfected with various  $\mu$  gene constructs (see Figures 1A and 2A) was assayed for the presence of specific  $\mu$  and H4<sup>ref</sup> transcripts by S1 nuclease mapping as described in Figure 1B and 1C. Lane M, Hpa II-cleaved pBR322 DNA. The position where a DNA fragment protected by  $\mu$ mRNA would migrate is indicated as  $\mu$ . The 155 nucleotide fragment visible in all lanes corresponds to the full-length DNA probe.

was transfected into MPC11 myeloma cells. Total RNA was isolated after 48 hr and analyzed for the presence of specific  $\mu$  transcripts by S1 nuclease mapping of RNA 5' ends (Figure 1B). Protected fragments, about 57 nucleotides in length, were generated (Figure 1B, lane 1), which corresponded to the same set of fragments protected by RNA from the hybridoma 17.2.25 (Figure 1B, lane 2). The initiation of transcription of the transfected  $\mu$  gene therefore appears to occur at the same site as in the endogenous rearranged V $\mu$  17.2.25 gene, which has been mapped to the nucleotide by Mung bean nuclease mapping (Grosschedl et al., 1985; see Figure 4B). The synthesis of transcripts derived from the modified H4 histone gene was measured

by S1 nuclease mapping using a 5' end-labeled DNA probe obtained from the wild-type H4 gene. Hybridization of total RNA from transfected MPC11 cells to the probe and S1 nuclease treatment generated a 99 nucleotide fragment, which was protected by RNA derived from the modified H4 gene (Figure 1C).

#### The Ig Heavy Chain Enhancer Can Be Functionally Replaced by the Moloney-Murine Leukemia Virus Enhancer

To investigate the role of the Ig heavy chain gene enhancer (Banerji et al., 1983; Gillies et al., 1983; Neuberger, 1983) in determining tissue specificity of gene expression and modulation of the transcription level during B cell differentiation, we first analyzed the effects of deleting the IgH enhancer or replacing it with a tissue nonspecific viral enhancer. Deletion of all J<sub>H</sub>/C<sub>μ</sub> intron DNA sequences between the Xba I sites (e.g., deletion mutant pμΔ1; Figure 1A) abolished detectable levels of μ gene transcription in transfected MPC11 myeloma cells (Figure 2B, lane 1). The same phenotype was observed with deletion mutant pμΔ2, which lacked the 1 kb Xba I<sub>1/2</sub> DNA fragment containing the enhancer (Figure 1A; data not shown). In contrast, the 3.3 kb Xba I<sub>2/3</sub> DNA fragment comprising the heavy chain gene switch-region between the enhancer and the C<sub>μ</sub> exons could be deleted (mutant pμΔ3; Figure 1A) without any phenotypic effect in MPC11 cells, implying that these DNA sequences are dispensable for heavy chain gene expression in myeloma cells (data not shown).

To replace the μ enhancer with a viral enhancer, we inserted each enhancer element 5' to the promoter and thus avoided structural differences in the mRNA precursor derived from each construct. Deletion mutant pμΔ1 was used as a recipient for the insertion of the enhancer elements. The μ enhancer residing in the 1 kb Xba I<sub>1/2</sub> fragment was inserted into the Hind III site 1 kb 5' to the promoter in either orientation (Figure 2A). Transfection of the DNA constructs, termed pμΔ1(μE1), with the μ enhancer in normal orientation, and pμΔ1(μE1\*), with the μ enhancer in reversed orientation, into myeloma MPC11 cells resulted in the accumulation of μ transcripts to a level 50% of that observed with the wild-type template (Figure 2B, lanes 2-4). The quantitation of μ gene transcription was performed by densitometric scanning of autoradiograms for transcript bands specific for μ heavy chain and H4 reference genes. The transcripts of the H4 reference gene were used to normalize the levels of specific μ mRNA (Table 1). The same number of μ transcripts was obtained from the mutant DNA constructs pμΔ1(μE2) and pμΔ1(μE2\*), which had a 700 bp Xba I/Eco RI DNA fragment comprising the IgH enhancer inserted 5' to the promoter (Figure 2A; data not shown).

A viral enhancer was obtained from the long terminal repeat (LTR) of Moloney murine leukemia virus (M-MuLV) and inserted into the Hind III site 5' to the V<sub>H</sub> promoter (Figure 2A). The enhancer of M-MuLV (Mo enhancer) is functional in fibroblast cells (Laimins et al., 1982). The functional assay of the resulting constructs pμΔ1(MoE) and pμΔ1(MoE\*) in myeloma MPC11 cells revealed that the viral enhancer in either orientation could activate tran-

Table 1. Relative Transcript Level for RNA from Mutant μ Genes and Hybrid Genes in Fibroblast, Pre-B, and Myeloma Cells

Gene Construct	mRNA	NIH/3T3	PD36	MPC11
Relative Transcript Level				
μ (wild type)	μ	<0.01	0.4	2.0
μΔ1	μ	<0.01	<0.01	<0.01
μΔ1(μE1)	μ	<0.01	0.2	1.0*
μΔ1(MoE)	μ	<0.01	0.2	0.5
(Mo/Mo)μ	Moμ	0.31	1.5	4.9
(Mo/Mo)gpt	Mogpt	0.52	0.27	1.0*
(μ/μ)gpt	μgpt	0.03	0.36	1.0*
(Mo/μ)gpt	μgpt	0.07	0.48	0.56
(Mo/μ*)gpt	μgpt	0.07	0.54	0.63

The structure of the gene constructs is shown in Figures 1A, 2A, 4A, and 5A. In the first four lines, the relative transcript level (RTL) of wild-type and mutant μ genes is indicated. μΔ1(μE1) and μΔ1(MoE) have the IgH enhancer or the M-MuLV enhancer inserted at the 5' end of an enhancerless μ gene. In the other lines, the RTL of various hybrid genes are presented. The enhancer/promoter elements are written in parentheses. Transcripts from the hybrid genes have either 30 nucleotide M-MuLV(Mo) sequences or 57 nucleotide μ sequences attached to their 5' ends. The RTL was determined for NIH/3T3 fibroblasts, PD36 pre-B cells, and MPC11 myeloma cells. For the determination of the RTL, various exposures of the autoradiograms were scanned densitometrically. To calculate the RTL of each gene construct, the experimental band intensity was divided by the intensity of the H4 histone reference signal. This ratio was normalized within each set of data to an arbitrarily chosen standard as value 1.

\* Standard value.

scription of the μ heavy chain gene in myeloma cells to 50% of the level obtained with the μ enhancer (Figure 2B, lanes 5 and 6). Thus, the tissue-nonspecific viral enhancer can substitute for the IgH enhancer in myeloma cells.

#### The Enhancer Does Not Solely Determine Tissue Specificity of Heavy Chain Gene Transcription

To examine the transcriptional activity of the μ gene containing the IgH or the Mo enhancer in lymphoid and in nonlymphoid cell lines, we first determined whether the tissue specificity of heavy chain gene transcription is maintained in our assay. When we transfected wild-type and mutant μ genes lacking the Ig enhancer into NIH/3T3 fibroblastic cells, virtually no μ transcripts could be detected from either template, suggesting that the gene is transcriptionally inactive in fibroblastic cells (Figure 2C, lanes 1-3). From the ratio of reference and endogenous H4 transcripts, we estimated the transfection efficiency of fibroblasts to be similar to that of myeloma MPC11 cells (not shown). Because we could detect 1% of the μ mRNA signal detected in myeloma cells, we conclude that the μ gene in fibroblasts is at least 100-fold less transcriptionally active than in myeloma cells (Table 1).

The heavy chain enhancer is inactive in fibroblastic cells when tested on tissue-nonspecific transcription units (Banerji et al., 1983; Gillies et al., 1983; Grosschedl and Baltimore, unpublished). If the heavy chain enhancer were the sole determinant for tissue specificity of gene expression, it should be possible to activate the transfected heavy chain gene in fibroblast cells by providing an enhancer that is functional in these cells. Transfection of the

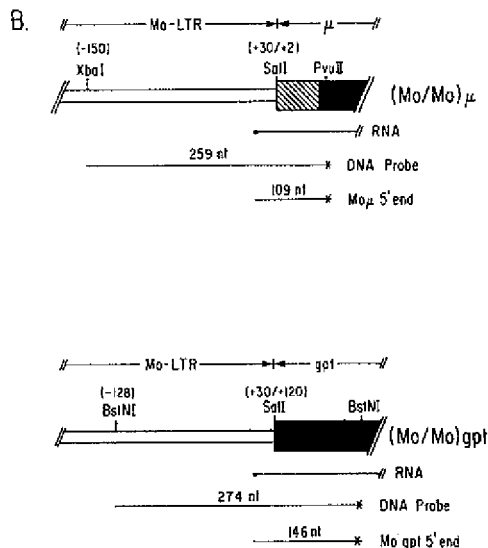
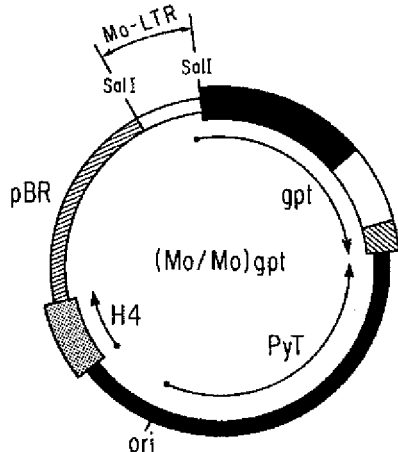
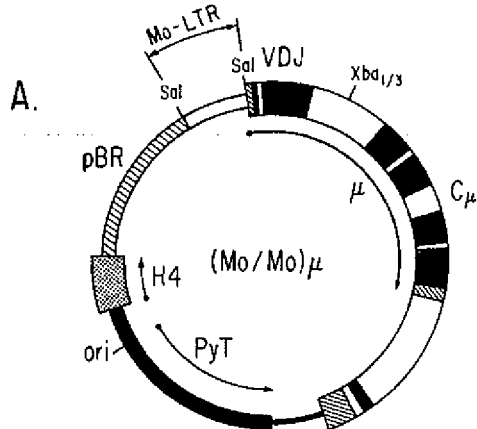


Figure 3. Intragenic  $\mu$  Sequences Regulate Tissue-Specific Ig Gene Expression

(A) Structure of hybrid genes consisting of M-MuLV LTR sequences linked to either  $\mu$  intragenic sequences or *gpt* gene sequences. (Top) the structure of the  $(Mo/Mo)\mu$  gene construct is shown comprised of the M-MuLV enhancer and promoter fused to  $\mu$  intragenic sequences that were deleted between the *Xba*I and *Xba*I<sub>3</sub> restriction sites. (Bottom) the plasmid  $(Mo/Mo)gpt$  is depicted. The *gpt* gene was derived from pSV2*gpt* (Mulligan and Berg, 1980) and is comprised of *gpt* coding sequences (filled wide bar) and an SV40-derived splice site (open box) plus polyadenylation site (hatched box).

(B) Scheme of S1 nuclease mapping of transcripts from the hybrid genes. The structure of the 5' ends of the  $(Mo/Mo)\mu$  gene (top) and the  $(Mo/Mo)gpt$  gene (bottom) are shown. Viral LTR sequences with their 3' ends terminating at +30 (relative to the viral transcription initiation site) were fused to  $\mu$  sequences at +2 (relative to the  $\mu$  mRNA cap site) and to *gpt* sequences at +120 (relative to the Hind III site upstream of the coding sequences, as defined in Mulligan and Berg, 1980), respectively. Restriction sites used for preparation of the 5' end-labeled, single-stranded DNA probes are indicated. The structures and the sizes of the DNA probes and protected DNA fragments are shown. Because the  $\mu$  and *gpt* transcripts derived from the hybrid genes contain 30 viral nucleotides attached to their 5' ends, the hybrid transcripts are termed *Mo $\mu$*  and *Mo*gpt**, respectively.

(C) Expression of  $(Mo/Mo)\mu$  and  $(Mo/Mo)gpt$  genes in various cell lines. Total RNA (20  $\mu$ g) from cells transfected with hybrid gene constructs was analyzed by S1 nuclease mapping. The positions of the DNA fragments protected by 5' terminal sequences of the hybrid *Mo $\mu$*  and *Mo*gpt** transcripts are indicated. The slower migrating fragments represent the full-length DNA probes. In the lower gel, the S1 nuclease analysis of H4 reference gene transcripts is shown. Lane M, Hpa II-cleaved pBR322 DNA as size marker.

DNA constructs  $p\mu\Delta 1(MoE)$  and  $p\mu\Delta 1(MoE^*)$ , in which the IgH enhancer had been replaced by the viral Mo enhancer, into NIH/3T3 fibroblastic cells did not, however, result in the accumulation of  $\mu$  transcripts to detectable levels (Figure 2C, lanes 6 and 7). Transcription of the modified H4 gene was used to control for the transfection efficiency (Figure 2C, bottom). Because a tissue-nonspecific enhancer was not sufficient to allow heavy chain gene activation in nonlymphoid cells, sequences in the remaining part of the Ig gene must limit  $\mu$  gene transcription in a tissue-specific manner.

### Intragenic Sequences Contribute to the Tissue Specificity of Gene Expression

To investigate whether the promoter and/or intragenic sequences contribute to the tissue specificity of the Ig transcription unit, we separated promoter and intragenic sequences and tested them individually. For the purpose of this study, we define  $\mu$  intragenic sequences as the transcribed region of the mutant  $\mu\Delta 1$  gene, including some downstream sequences, but lacking the IgH enhancer and most of the large  $V_HDJ_H/C_\mu$  intron (Figure 1A). We examined the role of  $\mu$  intragenic sequences in tissue-specific Ig gene expression by measuring the accumulation of RNA from a hybrid gene with  $\mu$  intragenic sequences linked to tissue-nonspecific transcriptional control signals in myeloma and in fibroblast cells. Murine retroviral LTR sequences from position -530 to +30 (relative to the mRNA cap site), including enhancer and promoter, were fused via synthetic Sal I DNA linkers to  $\mu$  intragenic sequences (Figure 3A). In this hybrid gene, termed (Mo/Mo) $\mu$ , the  $\mu$  intragenic sequences were obtained by deleting all 5'-flanking sequences of the mutant  $\mu\Delta 1$  gene (Figure 1A) upstream of position +2 (Figures 3A and 3B). For comparison, a similar hybrid gene, termed (Mo/Mo) $gpt$ , was constructed. This gene had viral LTR sequences linked to a tissue-nonspecific transcription unit consisting of the bacterial *gpt* gene and SV40-derived splice and polyadenylation sites (Figure 3A). Both hybrid genes were cloned into the short-term expression vector and transfected into MPC11 myeloma and NIH/3T3 fibroblast cells. Synthesis of specific transcripts was assayed by S1 nuclease mapping. For both genes, transcripts began at the expected site within the LTR and contained 30 viral nucleotides attached to their 5' ends (Figure 3B). Transcripts from the hybrid (Mo/Mo) $\mu$  gene were detected in both MPC11 and NIH/3T3 cells (Figure 3C, lanes 2 and 3). We calculated the transcription level of this hybrid gene in both cell types using transcription of the H4 reference gene for normalization (Table 1). MPC11 cells contained 16-fold more Mo $\mu$  RNA than did NIH/3T3 cells. In comparison, transcripts of the (Mo/Mo) $gpt$  gene were detected in myeloma cells at a 2-fold higher level than in fibroblasts (Figure 3C, lanes 4 and 6; Table 1). Therefore, intragenic sequences allowed for an 8-fold enhanced accumulation of transcripts from the (Mo/Mo) $\mu$  gene in myeloma cells, as compared with fibroblastic cells. This result suggests that  $\mu$  intragenic sequences contribute significantly to the tissue specificity of  $\mu$  gene expression.

### Tissue- and Cell-Type-Specificity of $V_H$ Promoter Sequences

Replacement of the Ig enhancer and 5'-flanking sequences of the  $\mu$  gene with viral transcription signals led to a partial activation of this gene in fibroblastic cells (Figure 3C, lane 2). In contrast, substitution of the IgH enhancer alone with a tissue-nonspecific enhancer did not allow for any detectable transcription in nonlymphoid cells (Figure 2C, lanes 6 and 7). These data suggest that 5'-flanking  $\mu$  gene sequences play a crucial role in determining tissue specificity of  $\mu$  gene transcription. To examine whether  $V_H$  promoter sequences are important for tissue-

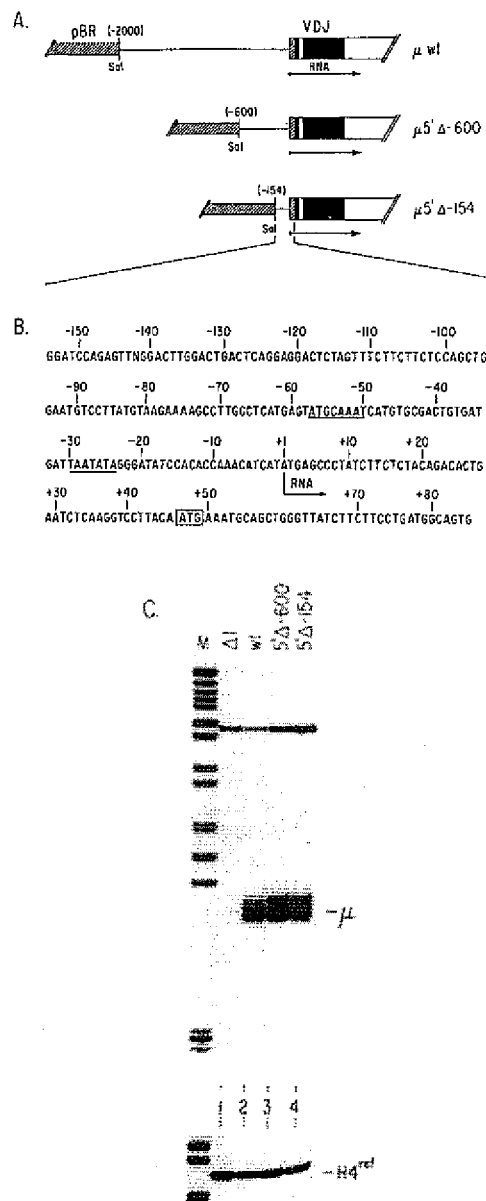


Figure 4. Delimitation of  $V_H$  Promoter Sequences

(A) Structure of 5' deletion mutants. The 5'-flanking sequences of the rearranged  $\mu$  gene are represented as thin lines. Noncoding  $V_H$ -derived sequences are drawn as a hatched area, exons as black, introns as open. The nucleotide positions of the boundaries of the rearranged  $V_HDJ_H$  region between -154 and +85 relative to the transcription initiation site. The mRNA cap site (Grosschedl et al., 1985) is indicated. The TATA consensus sequence and an octanucleotide sequence conserved among all known V regions (Parslow et al., 1984), are underlined. The initiation codon for translation is boxed. (C) Expression of 5' deletion mutants in MPC11 myeloma cells. MPC11 cells were transfected with wild-type and mutant  $\mu$  genes. Total RNA was isolated and analyzed for expression of the  $\mu$  genes and the H4 reference genes by S1 nuclease mapping as described in Figure 1. The positions of protected  $\mu$  and H4<sup>ref</sup> fragments are indicated. The upper band corresponds to the full-length DNA probe.

specific gene expression, we first delimited the 5' boundary of the functional promoter by constructing two mutant  $\mu$  genes lacking portions of 5'-flanking sequences. One deletion mutant,  $\mu\Delta 5'-600$ , was generated by fusing the Hinc II site at nucleotide position -600 from the site of transcription initiation onto the pBR322 vector sequences (Figure 4A). Another mutant,  $\mu\Delta 5'-154$  was obtained by deleting all sequences between the Sal I site of the vector and the Bam HI site at position -154 of the  $\mu$  gene. Both mutants contained the Ig enhancer in its normal position in the large intron between the  $V_HDJ_H$  and the  $C_\mu$  region. The 5' deletion mutants of the  $\mu$  gene were transfected into MPC11 myeloma cells, total RNA was isolated and analyzed for specific  $\mu$  and H4 transcripts. As a positive control, the wild-type  $\mu$  gene was used. Both mutant templates directed  $\mu$  gene transcription at a 2-fold higher level than did the wild-type DNA, indicating that the 5'-flanking sequences upstream of position -154 can be removed without deleterious effect on  $\mu$  gene transcription (Figure 4C, lanes 2-4). The 5' boundary of the  $V_H$  promoter therefore mapped downstream of nucleotide position -154.

To examine the role of the  $V_H$  promoter for tissue- and cell-stage-specificity of Ig gene transcription, we constructed a hybrid gene by joining  $V_H$  promoter sequences to *gpt* gene coding sequences. Two promoter fragments, with their 5' boundaries at nucleotide position -600 and -154, respectively, and their 3' boundaries at position +57, were isolated from the rearranged  $\mu$  gene. A Pvu II restriction site at position +57 was chosen as the 3' boundary of the promoter to retain DNA sequences around the initiation site of transcription. Because the  $V_H$  promoter of the rearranged heavy chain gene is virtually inactive without an enhancer, we ligated either the IgH enhancer or the tissue-nonspecific Moloney virus enhancer to the 5' end of the  $V_H$  promoter fragments. The enhancer/promoter regulatory elements were fused to the bacterial *gpt* gene containing SV40 splice and polyadenylation sites and were cloned into the short-term expression vector (Figure 5A). The resulting hybrid genes,  $(\mu/\mu)gpt$  and  $(Mo/\mu)gpt$ , have the  $\mu$  IgH enhancer and the Mo enhancer, respectively, linked to the long  $V_H$  promoter fragment. The  $(Mo/\mu)gpt$  gene construct contained the Mo enhancer and the short  $V_H$  promoter fragment. The hybrid genes were transfected into MPC11 myeloma cells, and transcription was assayed by S1 nuclease mapping (Figure 5B). Initiation of the *gpt* transcripts occurred at the normal initiation site within the  $V_H$  promoter fragments. Therefore, the *gpt* transcripts contained 57 nucleotides of the rearranged  $\mu$  gene attached to their 5' end. Few  $\mu gpt$  transcripts were synthesized from any of the hybrid gene (Figure 5C, lanes 4-6). The transcription level of the  $(\mu/\mu)gpt$  gene was 2-fold higher than that of the  $(Mo/\mu)gpt$  gene construct, indicating the slightly lower activity of the Moloney virus enhancer (cf. Figure 2B). Adjusting for the transfection efficiency indicated by the H4 reference gene transcription, the  $(Mo/\mu)gpt$  gene was as active as the  $(Mo/\mu)gpt$  gene (Table 1). The relatively low transcriptional activity of these hybrid genes in myeloma cells can be at least partially explained by the contribution of  $\mu$  intragenic sequences to gene expression in these cells. The  $\mu$  tran-

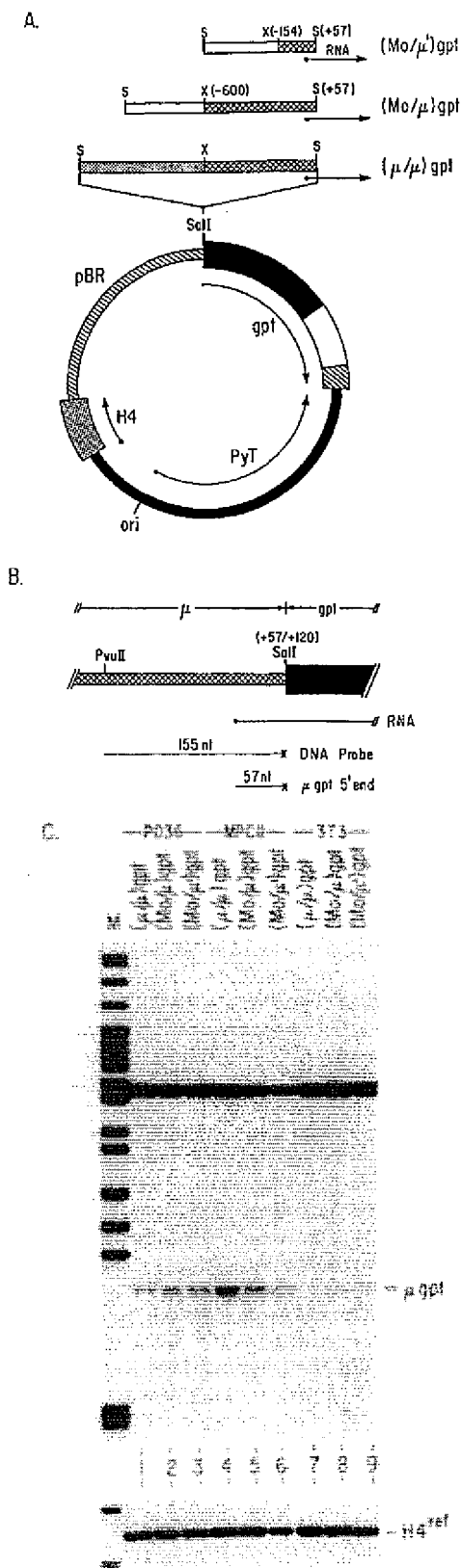
scripts derived from the hybrid  $(Mo/Mo)\mu$  gene accumulate in myeloma cells at a 5-fold higher level than do *gpt* transcripts obtained from the  $(Mo/Mo)gpt$  gene (Figure 3C, lanes 3 and 6; Table 1).

To determine the activity of the  $V_H$  promoter region in fibroblastic cells, we tested the hybrid genes in NIH/3T3 cells. A very small number of  $\mu gpt$  transcripts could be detected in total RNA from NIH/3T3 cells transfected with the  $(\mu/\mu)gpt$  gene (Figure 5C, lane 7). Replacement of the IgH enhancer with the tissue-nonspecific Moloney virus enhancer in the gene construct  $(Mo/\mu)gpt$  increased the *gpt* transcription level only 2-fold (Figure 5C, lane 8; Table 1). Using the H4 reference gene expression for normalization, we calculated the transcriptional activity of the  $(Mo/\mu)gpt$  gene to be about 10-fold higher in myeloma cells than in fibroblast cells (Table 1). These results imply that the  $V_H$  promoter region can confer tissue specificity to the bacterial *gpt* gene, even in the presence of a viral enhancer. The phenotype of the  $(Mo/\mu)gpt$  gene was identical with that of the  $(Mo/\mu)gpt$  gene construct (high expression in lymphoid cells, low expression in nonlymphoid cells; Figure 5C, lanes 5, 6, 8, and 9). Thus, the  $V_H$  promoter region between -154 and +57 appears to contain DNA sequences required for tissue-specific transcription.

#### Regulation of Ig Gene Expression during B Cell Differentiation

Although between pre-B and plasma cell stages the level of accumulated heavy chain transcripts increases 30- to 100-fold (Perry and Kelley, 1979), the rates of Ig heavy chain transcription between these cell types differ only by a factor of 2-5 (Mather et al., 1984). To examine whether Ig gene transcription depends on enhancer activity at the early stage of B lymphoid cell differentiation, we assayed transcription of wild-type and mutated  $\mu$  gene constructs in pre-B and in B cells.

As representative pre-B cells, we used the Abelson virus-transformed cell line PD36 as recipient for DNA transfection experiments. These cells have their endogenous  $\mu$  gene locus rearranged and are undergoing  $\kappa$  gene rearrangement (Lewis et al., 1982). Transfection of the wild-type  $\mu$  gene resulted in the accumulation of specific  $\mu$  transcripts at a level 5 times less than in MPC11 cells (Figure 6A, lane 1, Table 1). Thus, in the transient expression assay, the transfected Ig gene is only 5 times less active in pre-B cells than in myeloma cells. To test the enhancer requirement for  $\mu$  gene transcription in PD36 cells, we introduced the Ig enhancerless constructs  $\mu\Delta 1$  and  $\mu\Delta 2$  into these cells. No specific  $\mu$  transcripts could be detected by S1 nuclease mapping (Figure 6A, lanes 2 and 3), implying that the enhancer is required for  $\mu$  gene transcription at this early stage of the B cell lineage. Gene transcription could be restored to 50% of the wild-type level when the IgH enhancer or the Mo enhancer was inserted 5' to the  $V_H$  promoter (Figure 6A, lanes 5-8). As representative mature B cells we transfected WEHI 279.1 cells (Warner et al., 1979) with the various  $\mu$  gene constructs (Figure 6B). As in pre-B cells, an enhancer was required for transcription, but no differential activity between the Mo and IgH enhancers was evident.



Using the other gene constructs previously examined in MPC11 cells, we studied the sequence elements required for Ig gene expression in PD36 cells. The (Mo/μ)<sup>gpt</sup> and (Mo/μ)<sup>gpt</sup> genes were expressed at the same level in PD36 and MPC11 cells (Figure 5C, lanes 2, 3, 5 and 6; Table 1), suggesting that the V<sub>H</sub> promoter linked to the Mo enhancer is as active in pre-B cells as in terminally differentiated cells. Analysis of the transcriptional activity of the (μ/μ)<sup>gpt</sup> gene in PD36 cells revealed that transcripts from this gene accumulate at a level 3 times lower than in MPC11 cells (Figure 5C, lanes 1 and 4; Table 1). To test the influence of μ intragenic sequences on gene expression in pre-B cells, we measured the levels of transcripts derived from the (Mo/Mo)<sup>gpt</sup> and (Mo/Mo)<sub>μ</sub> genes in PD36 cells (Figure 3C, lanes 1 and 5). Transcripts from the (Mo/Mo)<sub>μ</sub> gene accumulated at a 5-fold higher level, as was found in MPC11 cells (Table 1), implying that μ intragenic sequences increase mRNA accumulation in both lymphoid cell types. Furthermore, taking into account the 2-fold lower transcriptional activity of the (Mo/Mo)<sup>gpt</sup> gene in PD36 cells, as compared with NIH/3T3 cells, the (Mo/Mo)<sub>μ</sub> gene appears to be 10-fold more active in pre-B cells than in fibroblastic cells (Table 1). Thus, we see no major difference in the requirement for regulatory Ig gene sequences in PD36 and in MPC11 cells.

## Discussion

### Regulation of Tissue-Specific Ig Gene Expression

Immunoglobulin heavy chain genes are transcriptionally active only in lymphoid cells, and their level of expression is regulated within this cell lineage (Perry and Kelley, 1979). From experiments involving transfection of rearranged genes into cultured cells (Gillies et al., 1983; Neuberger, 1983) and from germline gene transfer experi-

Figure 5. Tissue-Specificity of V<sub>H</sub> Promoter Sequences

(A) Schematic representation of hybrid gene constructs containing M-MuLV or IgH enhancer, V<sub>H</sub> promoter and gpt coding sequences. The various enhancer/promoter fragments used for fusion to gpt gene sequences are shown in linear form above the circular map. V<sub>H</sub> promoter fragments are drawn as crosshatched bars with their boundaries given in nucleotides relative to the initiation site of RNA synthesis (arrow). The μ IgH enhancer fragment is shown as a dotted bar and consists of the 700 bp Xba I/Eco RI DNA fragment (see Figure 2A) with the Eco RI site converted to a Sal I site. The viral Mo enhancer is represented by an open bar. Restriction sites are abbreviated as S for Sal I and X for Xba I. The structure of the gpt gene is identical with that shown in Figure 3A.

(B) Scheme of S1 nuclease mapping of RNA from hybrid genes. The structure of the 5' end of the hybrid genes is shown. The V<sub>H</sub> promoter region is drawn as a crosshatched bar, gpt coding sequences as a black box. The 3' boundary of the V<sub>H</sub> promoter fragment at nucleotide position +57, relative to the transcription initiation site, was ligated via a Sal I DNA linker to gpt sequences at +120 (relative to the Hind III site upstream of the coding sequences as defined in Mulligan and Berg, 1980). The structures and sizes of the DNA probe and the protected fragment are indicated.

(C) Analysis of RNA from hybrid genes in various cell types. Total RNA (50 μg) from various cell lines transfected with (μ/μ)<sup>gpt</sup>, (Mo/μ)<sup>gpt</sup>, and (Mo/Mo)<sup>gpt</sup> was analyzed by S1 nuclease mapping for specific μgpt and H4<sup>ref</sup> transcripts. The position of the DNA fragment protected by 5' terminal μgpt RNA sequences is indicated as μgpt. The slower migrating band represents the full-length DNA probe. Cell lines and constructs are indicated above each lane.

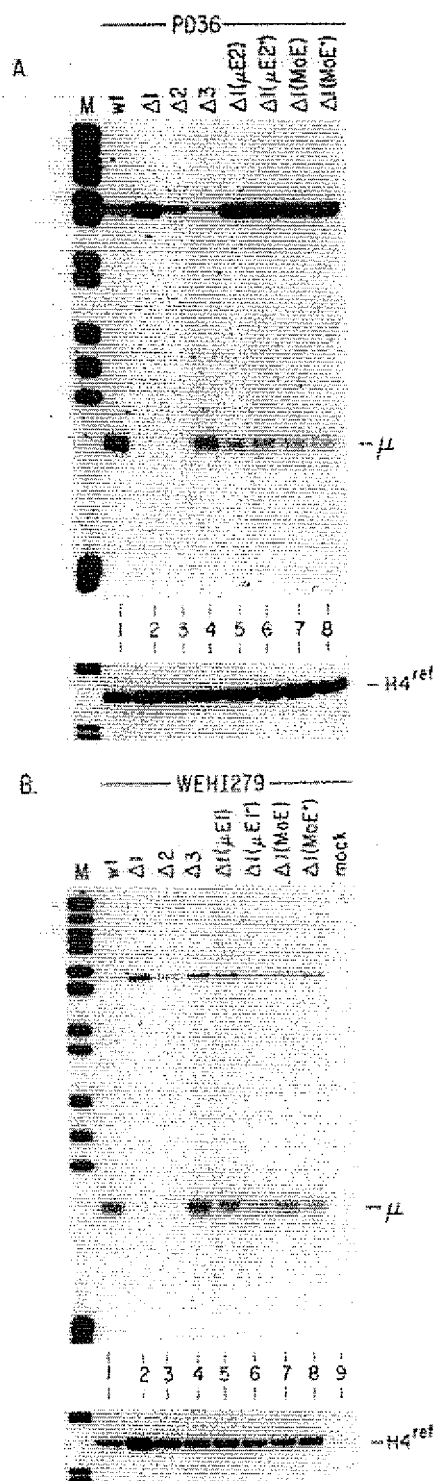


Figure 6. Transcriptional Activity of the Wild-Type and Mutant  $\mu$  Gene Constructs in Pre-B Cells (PD36) and in Mature B Cells (WEHI 279.1) (A) Analysis of 5' ends of specific  $\mu$  transcripts in transfected PD36 cells by S1 nuclease mapping. Total RNA (50  $\mu$ g) was hybridized to the  $\mu$  DNA probe and digested with S1 nuclease. For the analysis of H4<sup>ref</sup> transcripts, 5  $\mu$ g total RNA was used. (B) S1 nuclease analysis of specific  $\mu$  and H4 transcripts in total RNA from transfected WEHI 279.1 cells.

ments (Grosschedl et al., 1984), it has become clear that the elements controlling this regulation are located near the gene and are active in *cis*. We have studied the genetic basis for the tissue specificity of Ig heavy chain gene expression by dissecting the transcription unit into various elements, each of which contains regulatory information.

Virtually no expression of a transfected wild-type  $\mu$  gene has been detected in fibroblast cells. Because transfected heavy chain genes require an enhancer in order to be expressed at high levels in lymphoid cells (Gillies et al., 1983; Neuberger, 1983; this paper), the lack of Ig gene transcription in fibroblast cells could be formally explained by an inactivity of the enhancer. Indeed, the heavy chain gene enhancer is inoperative in fibroblastic cells, because it can activate heterologous genes in lymphoid cells but not in fibroblastic cells (Banerji et al., 1983; Gillies et al., 1983; Grosschedl and Baltimore, unpublished). Expression of the  $\mu$  heavy chain gene, however, remained lymphoid-specific when the Ig enhancer was replaced by the tissue-nonspecific enhancer from M-MuLV, implying that the IgH enhancer is neither the only, nor necessarily the dominant, regulatory element for the control of tissue specificity of Ig gene expression. Our experiments demonstrated that two additional regulatory sequences, the V<sub>H</sub> promoter region and the gene itself, also regulate the tissue specificity of expression.

#### The Role of the Ig Enhancer for Gene Expression

In myeloma cells, transcription of a transfected rearranged Ig heavy chain gene was shown to be dependent on the presence of an enhancer (Gillies et al., 1983; Neuberger, 1983; Figure 2B). We addressed the question of whether the enhancer is required for gene expression at earlier stages of cell differentiation by introducing the wild-type  $\mu$  gene and  $\mu$  gene constructs without an enhancer or with the Ig enhancer replaced by a viral enhancer into pre-B, mature B, and myeloma cell lines. No specific  $\mu$  transcripts were evident from the enhancerless  $\mu$  gene in any of the cells. Transcription of the  $\mu$  gene could be stimulated by the Ig enhancer at all three major stages of the B cell lineage, implying that the Ig enhancer is functional throughout B cell differentiation. Our results are in agreement with the detection of sequence-specific interactions of the IgH enhancer region with cellular factors at all three stages of B cell differentiation (Ephrussi et al., 1985). Therefore, the heavy chain enhancer appears to be tissue-specific but not cell-stage-specific. In this respect the Ig enhancer differs from the E $\beta$  gene enhancer, which was shown to be functional in mature B cells but inactive in myeloma cells (Gillies et al., 1984).

In some lymphoid T cell lines the Ig heavy chain locus is transcriptionally active, and "sterile"  $\mu$  transcripts are generated (Kemp et al., 1980; Alt et al., 1982). These sterile  $\mu$  transcripts initiate near the J<sub>H</sub> gene segments (Nelson et al., 1983). We tested the activity of the Ig enhancer after transfection of  $\mu$  gene constructs with and without enhancer into EL4 cells, a line that contains the "sterile"  $\mu$  transcripts, and demonstrated that the Ig enhancer could stimulate transcription in these cells (unpublished results).

This observation suggests that the enhancer may be involved in the developmental activation of the heavy chain locus at a very early stage of lymphoid cell differentiation.

Recently the functional importance of the Ig heavy chain enhancer for high level Ig gene expression has been brought into question by the observation that spontaneous deletions of heavy chain gene intron sequences, including the enhancer that occurred during growth of hybridoma cells, did not affect the level of Ig gene expression (Wabl and Burrows, 1984; Klein et al., 1984). A possible explanation for this observation is that the Ig enhancer is required at the initial stage of gene activation but is dispensable for maintenance of transcription. Our experiments do not rule out such a mechanism for enhancer activity because, by transfecting DNA into various cultured cells, we might mimic, in each case, the initial stage of gene activation. The results that the IgH enhancer can stimulate transcription at all major stages of the B cell lineage, however, indicated that the permissive conditions for Ig enhancer activity are present throughout B cell differentiation. Further support for the constitutive activity of the IgH enhancer in the lymphoid B cell lineage comes from the examination of the enhancer DNA sequences for physical interaction with *trans*-acting factors (Ephrussi et al., 1985). A persistent pattern of protection of specific nucleotides against methylation by dimethylsulphate suggested that a protein remains bound to the DNA during cellular differentiation.

#### The V<sub>H</sub> Promoter Region Controls Tissue-Specific Gene Expression

We have shown that the V<sub>H</sub> promoter region between nucleotide positions -154 and +57 can confer tissue specificity to the bacterial *gpt* gene. Transcripts of *gpt* initiating at V<sub>H</sub> promoter sequences accumulate in myeloma cells at a 10-fold higher level than in fibroblast cells. The V<sub>H</sub> promoter region can direct lymphoid-specific transcription even in the presence of a viral enhancer, suggesting that it acts as a dominant genetic element that can mask an active enhancer in nonlymphoid cells.

The precise location of the tissue-specific regulatory sequence within the V<sub>H</sub> promoter region between -154 and +57 has not yet been identified. Transcripts of the hybrid (Mo/ $\mu$ )*gpt* gene initiating at V<sub>H</sub> promoter sequences contain the 57 terminal nucleotides of  $\mu$  mRNA at their 5' end. Although these  $\mu$  sequences might be involved in the differential accumulation of such *gpt* transcripts in lymphoid versus nonlymphoid cells, a likely candidate for a tissue-specific control element is the octanucleotide ATG-CAAAT at position -50. DNA sequence comparison revealed that this octanucleotide is highly conserved in sequence and topology among all known variable regions of immunoglobulin genes (Parslow et al., 1984; Falkner and Zachau, 1984). A short DNA fragment that contains the inverted form of this conserved sequence appears to be possibly crucial, by functional tests, for  $\kappa$  gene transcription in myeloma cells (Falkner and Zachau, 1984; Bergman et al., 1984). Interestingly, the octanucleotide sequence is also present in the heavy chain enhancer (Falkner and Zachau, 1984), which might indicate that this

sequence could be involved in conferring tissue specificity in both promoter and enhancer elements. Such a mechanism would allow for the tissue specificity of multiple regulatory elements by one *trans*-acting factor. The occurrence of multiple binding sites has been described for the glucocorticoid receptor in the mouse mammary tumor virus genome (Scheidereit et al., 1983; Payvar et al., 1983).

#### Intragenic Sequences Contribute to Tissue-Specific Gene Expression

We have demonstrated that  $\mu$  heavy chain gene transcripts initiating at tissue-nonspecific viral transcriptional control elements accumulate in lymphoid and in nonlymphoid cells at different levels. Thus it appears that  $\mu$  intragenic sequences are involved in the enhanced accumulation of their transcripts in lymphoid cells. Several mechanisms can be considered to account for this tissue-specific regulation of  $\mu$  transcript levels by intragenic sequences. First, it is possible that sequences within the rearranged  $\mu$  gene, which lacks most of the VDJ/C $\mu$  intron sequences, regulate the rate of transcription initiation. Intragenic sequences were demonstrated to control the transcription of genes transcribed by RNA polymerase III (Bogenhagen et al., 1980; Sakonju et al., 1980; Galli et al., 1981). Second, splicing of pre-mRNA might be controlled in a tissue-specific fashion, thus leading to different amounts of mature stable RNA in different cell types. An example for this kind of regulation might be the troponin T gene; the differential splicing of its pre-mRNA is most likely regulated by intragenic sequences (Medford et al., 1984). A third mechanism would be a regulation of the transcript level by differential stability of the mRNA, as shown for some liver-specific mRNAs (Jefferson et al., 1984).

The functional importance of intragenic sequences for gene expression has been recently demonstrated for other genes transcribed by RNA polymerase II. Expression of the chicken *tk* gene in differentiating muscle cells is regulated by gene-internal sequences (Merrill et al., 1984). The stimulation of  $\beta$  globin expression in murine erythroleukemia cells by dimethyl sulphoxide and in HeLa cells by enhancers was also shown to be determined by intragenic sequences (Charnay et al., 1984; Wright et al., 1984; Treisman et al., 1983). The mechanism by which these intragenic sequences regulate gene expression has yet to be established for any gene.

Our evidence therefore implicates three elements in controlling the tissue-specific expression of an IgH chain. The enhancer acts only in lymphoid cells but can stimulate gene expression at all stages of lymphoid cell development. The promoter provides for 10-fold greater expression in lymphoid versus nonlymphoid cells but is not differentially active at different stages of B lymphoid cell development. Intragenic sequences provide for 8-fold greater synthesis in lymphoid cells than in fibroblasts and may also be the site for regulating cell-stage-specific differences in mRNA accumulation. Because most of the cell-stage-specific regulation of the  $\mu$  transcript level is posttranscriptional (Mather et al., 1984), intragenic se-

quences may regulate pre-mRNA splicing, RNA transport, or mRNA stability.

#### Cell-Stage-Specific Ig Gene Regulation during B Cell Differentiation

Measurements of the level of cytoplasmic  $\mu$  mRNA derived from endogenous Ig genes in pre-B and myeloma cells suggested a 30- to 100-fold greater accumulation of  $\mu$  transcripts in myeloma cells (Perry and Kelley, 1979). Cytoplasmic  $\mu$  mRNA from stably transfected pre-B and myeloma cells were found to accumulate at a 20-fold higher rate in myeloma cells (Neuberger, 1983). In the short-term transfection assay, we observed only a 5-fold difference in the  $\mu$  transcript level between pre-B and myeloma cells. We cannot account for this apparent discrepancy between in vivo data and data on transfected cells but two reasons might be considered. There is evidence that the ratio of nuclear RNA to cytoplasmic RNA is 50-fold higher in pre-B cells, as compared with myeloma cells (Perry and Kelley, 1979). Because we used total RNA for the analysis of the  $\mu$  transcript level, rather than cytoplasmic RNA, the quantitative difference of  $\mu$  transcripts between the two cell stages might be diminished. Another reason might be a consequence of the experimental system itself. We assayed total RNA from transfected cells two days after transfection. The early region of polyoma allows for the amplification of the DNA template, and the majority of the transcripts might be synthesized relatively late in the incubation period. Hence, a differential posttranscriptional regulation between pre-B and myeloma cells may not be seen to its full extent. Indeed most of the quantitative difference in the transcript level between pre-B and myeloma cells was found to be caused by posttranscriptional regulation (Mather et al., 1984).

Whatever the explanation for the lack of a quantitative difference in  $\mu$  mRNA accumulation between pre-B cells and plasma cells, the various gene constructs show qualitatively similar behavior in the two cell types. Therefore,  $\mu$  gene expression at all stages of B lymphoid cell differentiation is probably regulated by the same set of DNA elements located in the enhancer, promoter, and intragenic segments.

#### Experimental Procedures

##### Construction of Plasmids

The DNA construct referred to in this paper as  $p\mu$  is the same as the one described in Grosschedl et al. (1984) with the exception that the early region of polyoma virus (position 4631 to 2962; Griffin et al., 1980) has been inserted between the immunoglobulin gene and histone H4 gene. To generate the enhancer insertion mutations, a subclone of the rearranged  $\mu$  gene was constructed. The Sal I-Xho I DNA fragment of  $p\mu\Delta 1$  (see Figure 1A) harboring the VDJ region was cloned into a Sal I-Xba I-cleaved pUC13 vector that had no Hind III site. The resulting subclone was linearized with Hind III 1 kb upstream of the  $V_H$  transcription initiation site, and various DNA fragments carrying IgH or M-MuLV enhancer elements were linked with Hind III linkers and inserted into the Hind III site.

For the construction of the hybrid (Mo/Mo) $\mu$  gene, the plasmid  $p\mu\Delta 1$  was partially cleaved with Nde I, blunt-ended and ligated with synthetic Sal I linkers. The plasmid that had the Nde I site at position +2 (relative to the  $\mu$  mRNA cap site) converted to a Sal I site, was cleaved with Sal I and recircularized under dilute conditions thus deleting all  $\mu$  5'-

flanking sequences. A Cla I/Sma I DNA fragment containing LTR sequences of M-MuLV was ligated to Sal I linkers and inserted into the Sal I site of this  $p\mu\Delta 1$  gene construct lacking all  $\mu$  5'-flanking sequences.

The hybrid  $gpt$  genes were constructed by the following scheme: pSV2 $gpt$  (Mulligan and Berg, 1980) was cleaved with Bgl II at position +120 of the  $gpt$  gene (counting the nucleotides from the Hind III site) and with Bam HI downstream of the SV40-derived splice and polyadenylation site. The DNA fragment containing the  $gpt$  gene was blunt-ended and ligated to Sal I linkers. The Sal I-linked  $gpt$  DNA fragment was then ligated with Sal I-Xho I-cleaved vector DNA containing the H4 gene and the polyoma early region. The plasmid with a unique Sal I site at the 5' end of the  $gpt$  gene was used for further constructions. To prepare the large  $V_H$  promoter fragment, a Hind III-Stu I DNA fragment containing the 5' half of the  $V_{H2.25}$  gene and 5'-flanking sequences was isolated from the rearranged  $\mu$  gene. The DNA fragment was partially cleaved with Pvu II and ligated with Sal I linkers. After recutting with Sal I, the  $V_H$  promoter DNA fragment, with the Pvu II site at position +57 (relative to the mRNA cap site) converted to a Sal I site, was isolated. This DNA fragment was cleaved with Hinc II at position -600 and was ligated with Xba I linkers. After recutting the fragment with Xba I, the Xba I-Sal I-linked  $V_H$  promoter fragment was purified by gel electrophoresis and was cloned into pUC13. The small  $V_H$  promoter fragment was obtained by converting the Bam HI site at position -154 within the large  $V_H$  promoter fragment into an Xba I site. The Xba I-Sal I-linked promoter fragments were ligated with Xba I-Sal I-linked IgH- or Moloney virus-enhancer fragments and cloned into the Sal I site of the  $gpt$  gene plasmid.

##### Cell Culture and DNA Transfection

MPC11 myeloma cells were grown in DME supplemented with 5% calf serum and 5% fetal calf serum. NIH/3T3 cells were grown in DME containing 10% calf serum. Other lymphoid cells were grown in RPMI containing 10% fetal calf serum and 50  $\mu$ M 2-Mercaptoethanol.

For transfections,  $10^7$  lymphoid cells were washed with TS (8 g/l NaCl, 0.38 g/l KCl, 0.1 g/l  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , 3.0 g/l Tris, 0.1 g/l  $\text{MgCl}_2$ , 0.1 g/l  $\text{CaCl}_2$ ), pH 7.4 (Sompayrac and Danna, 1981), and were resuspended in 1.5 ml TS containing DNA at 2  $\mu$ g/ml and DEAE-dextran (Pharmacia;  $M_n \times 10^5$ ) at 0.5 mg/ml. The mixture was kept for 20 min at room temperature. Tissue culture medium (15 ml) containing 0.1 mM Chloroquine diphosphate (Sigma; Luthman and Magnusson, 1983) was added, and after 60 min at 37°C the Chloroquine-medium was replaced with normal tissue culture medium. The cells were harvested after 42-48 hr incubation, and total RNA was isolated and purified as described (Chirgwin et al., 1979; Gilsin et al., 1974).

##### S1 Nuclease Analysis

From 5 to 50  $\mu$ g total RNA was hybridized with single-stranded  $^{32}\text{P}$  5'-end-labeled DNA fragments at 2.5  $\mu$ g/ $\mu$ l as described (Hentschel et al., 1980). tRNA was used to adjust the amount of total RNA to at least 25  $\mu$ g. The hybridization temperatures were 38°C for the  $\mu$  DNA probe and 50°C for the H4, Mo $gpt$  and Mo $\mu$  DNA probes. The hybrids were digested with 50 units of S1 nuclease (PL Biochemicals) at 37°C for 1 hr. The protected DNA fragments were analyzed by electrophoresis through 8% polyacrylamide-urea gels.

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# Multiple Nuclear Factors Interact with the Immunoglobulin Enhancer Sequences

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## Summary

To characterize proteins that bind to the immunoglobulin (Ig) heavy chain and the  $\kappa$  light chain enhancers, an electrophoretic mobility shift assay with end-labeled DNA fragments was used. Three binding proteins have been found. One is NF-A, a factor found in all tested cell types that binds to the octamer sequence found upstream of all Ig variable region gene segments and to the same octamer in the heavy chain enhancer. The second, also ubiquitous, protein binds to a sequence in both the heavy chain and the  $\kappa$  enhancers that was previously shown to be protected from methylation *in vivo*. Other closely related sites do not compete for this binding, implying a restriction enzyme-like binding specificity. The third protein binds to a sequence in the  $\kappa$  enhancer (and to an identical sequence in the SV40 enhancer) and is restricted in its occurrence to B cells.

## Introduction

Immunoglobulin (Ig) gene expression is governed by three types of tissue-specific regulatory sequences (Grosschedl and Baltimore, 1985)—the promoter (Falkner and Zachau, 1984; Bergman et al., 1984; Mason et al., 1985; Gopal et al., 1985; Foster et al., 1985; Picard and Schaffner, 1985), the enhancer (Gillies et al., 1983; Banerji et al., 1983; Neuberger, 1983; Mercola et al., 1983; Queen and Baltimore, 1983; Queen and Stafford, 1984; Picard and Schaffner, 1984), and, at least in the case of the  $\mu$  heavy chain gene, by an intragenic sequence as well (Grosschedl and Baltimore, 1985). Within the upstream promoter region, sequence comparisons (Parslow et al., 1984) followed by deletion analysis have indicated the importance of an octameric motif (ATTGTCAT), located at a characteristic distance upstream of all sequenced variable region genes (Falkner and Zachau, 1984; Bergman et al., 1984; Mason et al., 1985). We have recently reported the detection of a nuclear factor that interacts with this sequence (Singh et al., 1986). Enhancers were defined in viruses as regulatory sequences that can potentiate transcription from a variety of promoters in a distance- and orientation-independent manner, but the Ig enhancers were the first found to be tissue-specific as well. It has been assumed that the action of these *cis* regulatory elements must be mediated by *trans*-acting factors, and there is now some experimental evidence to support this conjecture. Schöler and Gruss

(1984) and Mercola et al. (1985) have carried out *in vivo* competition experiments which imply that the presumed factor can be functionally titrated by cotransfection of increasing amounts of enhancer sequences. Also, Ephrussi et al. (1985) and Church et al. (1985) have obtained footprints of a putative tissue-specific factor on the heavy chain enhancer in living cells and nuclei. Recently, a substantial advance has also been made by the development of enhancer-dependent *in vitro* transcription systems (Sassone-Corsi et al., 1984; Wildeman et al., 1984; Sergeant et al., 1984; Schöler and Gruss, 1985). Competition experiments carried out *in vitro* (Schöler and Gruss, 1985; Sassone-Corsi et al., 1985) have further indicated that enhancer function may be mediated by *trans*-acting factors. To understand the mechanisms of enhancer function and their role in the activation of tissue-specific genes, we have searched for the presence of such factors in nuclear extracts.

We report here interaction of factors with Ig  $\mu$  and  $\kappa$  enhancer sequences as detected by an electrophoretic mobility shift assay (Fried and Crothers, 1981; Garner and Revzin, 1981). This technique, based on the fact that nucleoprotein complexes are resolved from uncomplexed DNA by electrophoresis through polyacrylamide gels in low ionic strength buffers, has been elegantly used for the kinetic and equilibrium analysis of a number of prokaryotic DNA binding proteins (Hendrickson and Schlieff, 1984, 1985; Fried and Crothers, 1984a, 1984b; Bushman et al., 1985). More recently it has been used to detect and purify eukaryotic proteins that were believed to recognize specific DNA sequences (Strauss and Varshavsky, 1984; Piette et al., 1985; Carthew et al., 1985; Levinger, 1985; Singh et al., 1986). Because a functional enhancer may consist of multiple protein-binding DNA segments, we have dissected the enhancer into small fragments retaining only one or two binding sites for sequence-specific proteins. In this way, at least three different Ig enhancer-binding proteins have been identified.

## Results

The fully functional  $\mu$  enhancer is included in a 700 bp XbaI-EcoRI fragment from the intron between  $J_H$  and  $C_H$ . This fragment can be further subdivided into a 400 bp XbaI-PstI fragment ( $\mu 400$ ) and a 300 bp PvuII-EcoRI fragment ( $\mu 300$ ). Transient transfection assays have shown that 30%–50% of the tissue-specific enhancer activity is retained in  $\mu 300$ , whereas there is no detectable activity in  $\mu 400$  (Grosschedl and Baltimore, 1985). We have used an electrophoretic mobility shift assay to investigate protein factors that interact with the  $\mu$  enhancer. In outline, the assay involves incubating end-labeled, specific DNA fragments plus unlabeled, nonspecific DNA for 30 min at room temperature with nuclear extracts made from tissue culture cells (Dignam et al., 1983). Protein-DNA complexes are then separated from free DNA by electrophoresis through a low ionic strength polyacrylamide gel and

visualized by autoradiography. When the functional 300 bp enhancer fragment ( $\mu 300$ ) was used in such an assay, a DNA-protein complex migrating more slowly than free DNA was observed with extracts derived from the human B lymphoma cell line EW (Figure 1B, lanes 1 and 2; the complex is indicated by the arrow). To show that this new band represented a specific complex, we carried out binding reactions in the presence of varying amounts of non-radioactive competitor fragments (Figure 1B, lanes 3-11). When  $\mu 300$  was added as the competitor fragment (Figure 1B, lanes 3-5), the complex band was completely lost with 200 ng of competitor (lane 5), whereas the adjacent  $\mu 400$  fragment (lanes 6-8) or the 450 bp fragment containing the  $\kappa$  light chain enhancer (lanes 9-11) yielded, at most, minor competition even at the highest concentrations used. The slight increase of the specific complex caused by the  $\kappa$  enhancer fragment (Figure 1B, compare lanes 9 and 2) could be due to its binding of factors common to both enhancers (described below), thus leaving more of the labeled fragment available to bind to a  $\mu$ -specific factor.

#### Localization of Heavy Chain Enhancer Binding

To define the complex detected with  $\mu 300$  more precisely, we further dissected this fragment by digestion with *AluI*, *HinfI*, and *DdeI*, generating a number of 50-70 bp fragments called  $\mu 50$ , ( $\mu 60$ )<sub>2</sub> (a mixture of  $\mu 60-1$  and  $\mu 60-2$ ), and  $\mu 70$  (Figure 2A). Binding reactions were carried out with each of these fragments using EW nuclear extracts in the presence of increasing amounts of the nonspecific competitor poly d(1C) (Figure 2B). Fragment  $\mu 50$  formed a major complex band (Figure 2B, lanes 2-4) that was barely decreased even in the presence of 3.6  $\mu$ g of poly d(1C) (lane 4). The mixture of the two 60 bp fragments did not yield a discrete complex band (Figure 2B, lanes 6-8). Finally the  $\mu 70$  fragment gave three faint, but discrete, nucleoprotein complex bands (Figure 2B, lane 10); the lower one was again barely affected by 3.6  $\mu$ g of non-specific carrier poly d(1C) (lane 12). The complex generated with  $\mu 50$  was specifically competed away by inclusion of 50 ng of  $\mu 300$  (of which  $\mu 50$  is a part) (Figure 2C, compare lanes 3 and 2) or a  $\kappa$  promoter fragment (lane 7) in the binding reaction but not by inclusion of corresponding amounts of  $\mu 400$  (lane 4), of the SV40 enhancer (lane 5), or of a fragment containing the  $\kappa$  enhancer (lane 6). This result implies that the  $\mu 50$  complex is generated by interaction of the DNA with a previously described factor, NF-A (previously referred to as IgNF-A; Singh et al., 1986), that recognizes a conserved octanucleotide, ATTTGCAAT, found both in the promoters of all sequenced immunoglobulin genes and within this subfragment of the heavy chain enhancer. We shall refer to this motif as the O sequence.

The complex observed with  $\mu 70$  was specifically competed away by only the  $\mu 300$  fragment (Figure 2D, compare lanes 3 and 2) and to some extent by the  $\kappa$  enhancer (data not shown), but was not at all competed away by either the Moloney murine leukemia virus enhancer (data not shown), the SV40 enhancer (data not shown), or the  $\mu 400$  fragment (compare lanes 4 and 2). Furthermore,

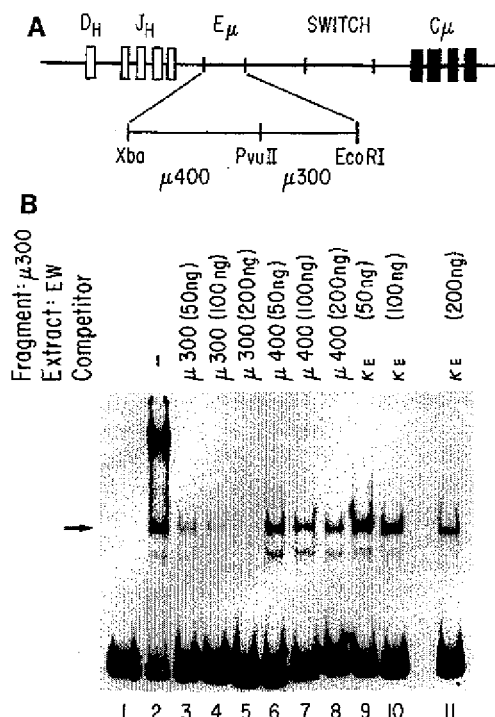


Figure 1. Factor Binding to the  $\mu$  Enhancer

(A) Schematic representation of the germ line immunoglobulin heavy chain locus. The  $\mu$  enhancer ( $E_{\mu}$ ) has been localized to a 700 bp *XbaI*-*EcoRI* fragment from within the  $J_{\mu}$ - $C_{\mu}$  intron (Grosschedl and Baltimore, 1985). When further dissected by cutting at the *PvuII* site, 30%-50% of the enhancer function is recovered in the 300 bp *PvuII*-*EcoRI* fragment ( $\mu 300$ ), whereas the *XbaI*-*PvuII* fragment ( $\mu 400$ ) does not carry any detectable enhancer function by transient transfection assay (Grosschedl and Baltimore, 1985).

(B) Electrophoretic mobility shift assay of  $\mu 300$ . End-labeled  $\mu 300$  (10,000 cpm, 0.5 ng) was incubated with 8  $\mu$ g of a nuclear extract derived from the human B lymphoma EW in the presence of 3.6  $\mu$ g of poly d(1C) and various amounts of nonradioactive competitor DNA fragments as noted, followed by electrophoresis through a low ionic strength polyacrylamide gel. The specific nucleoprotein complex is indicated by the arrow. Lane 1, free DNA fragment. Lane 2, binding reaction in the absence of competitor DNA. Lanes 3, 4, 5 are 50, 100, and 200 ng of  $\mu 300$  fragment included during binding. Lanes 6, 7, 8 are 50, 100, and 200 ng of  $\mu 400$  fragments included during binding. Lanes 9, 10, 11 are 50, 100, and 200 ng of a  $\kappa$  enhancer fragment ( $\kappa E$ ) included during binding. The  $\kappa$  enhancer fragment was excised from a plasmid that contained the *AluI*-*AluI* segment of the  $J_{\kappa}$ - $C_{\kappa}$  intron described by Picard and Schaffner (1984) cloned into the *SmaI* site of pUC 13 and was a kind gift of N. E. Speck.

competition experiments with subfragments from within  $\mu 300$  showed that this complex could not be competed away by either  $\mu 50$  (Figure 2D, lanes 5 and 6), ( $\mu 60$ )<sub>2</sub> (lanes 7 and 8), or  $\mu 170$  (lanes 11 and 12), but only by itself (lanes 9 and 10). Thus the dissection of  $\mu 300$  revealed two distinct and specific regions of binding, one in  $\mu 50$  (apparently the O sequence) and one in  $\mu 70$ .

Ephrussi et al. (1985) and Church et al. (1985) have used methylation protection experiments to define a set of G residues within the heavy chain enhancer that are specifically resistant to methylation by dimethyl sulfate (DMS) in B cells or B cell nuclei. This result led to the proposal that

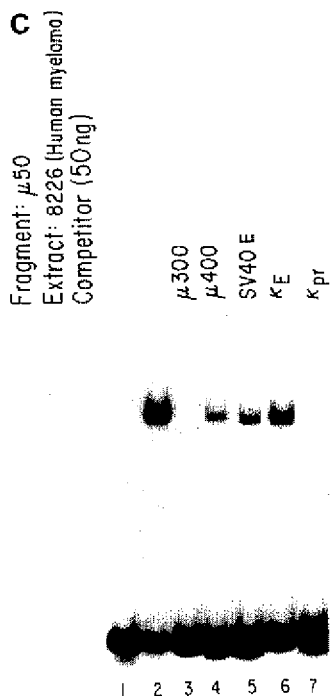
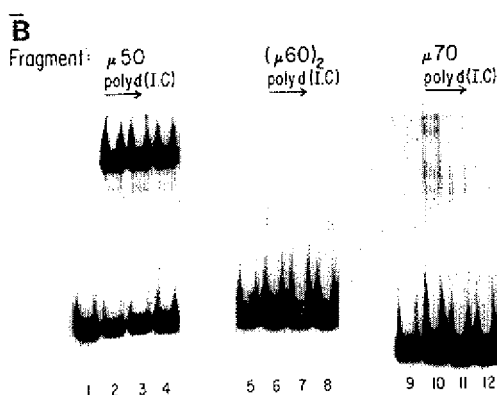
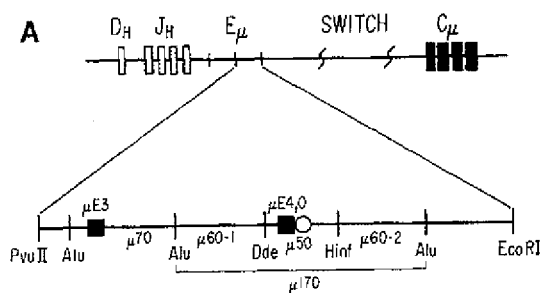
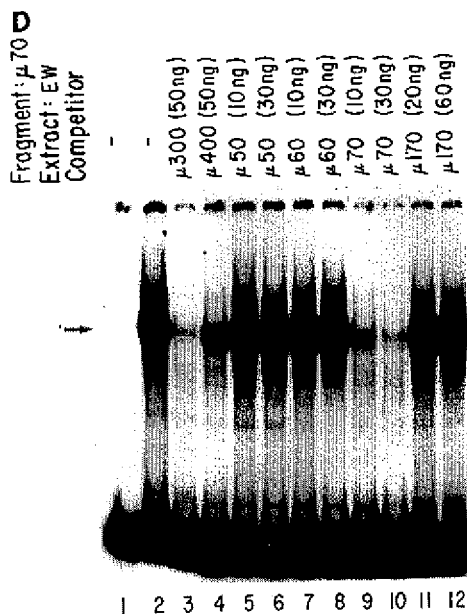


Figure 2. Dissection of the  $\mu 300$  Fragment to Localize Factor-Binding Sites

(A) Schematic representation. For further dissection, the  $\mu 300$  segment was cleaved with *AluI*, *DdeI*, and *HinfI* to generate the fragments named  $\mu 50$ ,  $\mu 70$ , and  $\mu 60$  (there are two fragments of approximately 60 bp obtained by restricting the large *AluI*-*AluI* piece [ $\mu 170$ ] with *DdeI* and *HinfI*). The black boxes represent regions of the  $\mu$  enhancer found



to be resistant to methylation by DMS *in vivo*. Four homologous domains have been identified earlier (Ephrussi et al., 1985), which we refer to as  $\mu E1$ - $\mu E4$ . Of these, only two,  $\mu E3$  and  $\mu E4$ , are present in the fragments being analyzed and are located in  $\mu 70$  and  $\mu 50$ , respectively. The open circle (O) refers to the octamer sequence (ATTG-CAT), which has been found in the promoters of all sequenced  $V_H$  and  $V_L$  genes and is also found in the  $\mu$  enhancer. The central G residue in this sequence was also found to be resistant to methylation in whole cells (Ephrussi et al., 1985).

(B) Binding analysis of subfragments of  $\mu 300$ . In each set of four lanes, the first shows the free fragment and the next three shows the effect of carrying out a binding analysis in the presence of increasing amounts (1.2, 2.4, and 3.6  $\mu$ g, respectively) of the nonspecific competitor poly d(I.C) and a constant amount (8  $\mu$ g) of protein derived from the EW cell line. ( $\mu 60$ )<sub>2</sub> represents a mixture of the two 60 bp fragments. Labeled fragments are as follows: lanes 1-4,  $\mu 50$ ; lanes 5-8, ( $\mu 60$ )<sub>2</sub>; lanes 9-12,  $\mu 70$ .

(C) Competition experiments to show that the complex formation on  $\mu 50$  is sequence-specific. Binding reactions were carried out in a final 15  $\mu$ l volume with 8  $\mu$ g of a nuclear extract derived from the human myeloma cell line 8226 with 0.1-0.3 ng of end-labeled probe ( $\sim 12,000$  cpm) and 2.5  $\mu$ g poly d(I.C). Lane 1, free fragment; lane 2, no competitor DNA added; lanes 3-7, binding reactions in the presence of 50 ng of different competitor fragments (as noted above the lanes) added prior to addition of the nuclear extract.  $\kappa pr$  is a 300 bp *Sfi*NI-*Sfi*NI fragment derived from the promoter region of the MOPC 41  $\kappa$  gene. It extends from position -330 to position -30 relative to the cap site (Queen and Baltimore, 1983). The conserved octanucleotide sequence (ATTG-CAT) is located between base pairs -59 and -66. SV40E is a 170 bp fragment containing both 72 bp repeats of SV40 from the *Hpa*II site on the late side.  $\kappa E$  is a fragment containing the  $\kappa$  enhancer (see Figure 1B for details).

(D) Competition experiments showing that complex formation on  $\mu 70$  is sequence-specific. Lane 1, free fragment (0.2-0.3 ng, 10,000 cpm); lane 2, binding reaction in a final volume of 15  $\mu$ l in the presence of 1.5  $\mu$ g of poly d(I.C) and 12  $\mu$ g of nuclear extract derived from the cell line EW; lanes 3-12, binding reactions as described for lane 2, but also containing unlabeled competitor DNA derived from the  $\mu$  enhancer in the amounts shown above each lane.

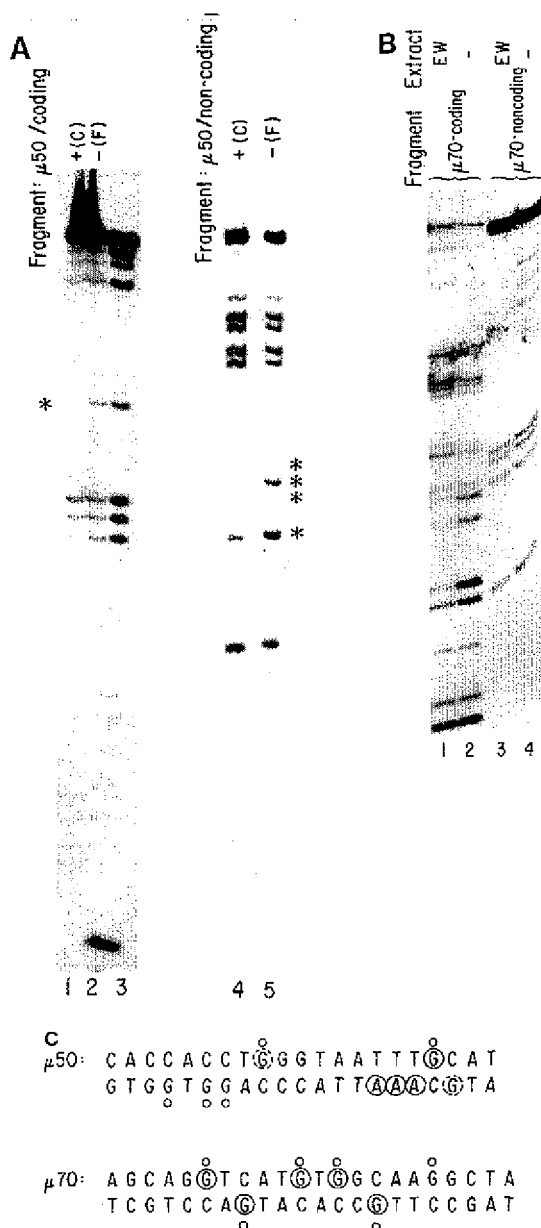


Figure 3. Methylation Interference Experiments to Define the Binding Sites of the Proteins That Interact with  $\mu 50$  and  $\mu 70$

The asterisks indicate the location of G residues whose methylation by DMS specifically inhibits the binding of a factor to its cognate sequence.

(A) The  $\mu 50$  fragment was end-labeled at the DdeI site (0.2–0.3 ng/10,000 cpm) on the coding strand. A typical preparative reaction was done with 80,000–100,000 cpm in an EW nuclear extract. Lane 1, nucleoprotein complex band (C) analyzed after elution from a preparative, low ionic strength polyacrylamide gel and piperidine treatment. Lane 2, free fragment band (F) from the same binding reaction. Lane 3, G ladder generated from end-labeled  $\mu 50$ , not exposed to any proteins. Analysis of the noncoding strand after labeling at the HinfI site. Lane 4, nucleoprotein complex band (C). Lane 5, free fragment band (F) from the same binding reaction.

(B) The AluI–AluI  $\mu 70$  fragment was subcloned into the SmaI site of pUC 13 (p $\mu 70$ ). The plasmid was restricted with BamHI and end-labeled for coding strand analysis or restricted with EcoRI and end-

labeled for noncoding strand analysis. Binding reactions were carried out in EW nuclear extracts. Lanes 1 and 2, analysis of the G residues on the coding strands of the complex band (lane 1) and the free fragment band (lane 2) obtained after a preparative binding and low ionic strength gel electrophoresis. Lanes 3 and 4, analysis of the G residues on the noncoding strand in the nucleoprotein complex band (lane 3) and the free fragment band (lane 4) following preparative binding and low ionic strength gel electrophoresis.

(C) Summary of the methylation interference (in vitro) and the methylation protection (in vivo) experiments used to define protein binding sites within the  $\mu$  enhancer: the relevant regions of the  $\mu 50$  and  $\mu 70$  fragments are shown with the coding strand on top in the orientation in which they appear in the  $\mu$  enhancer. The circles above the letters show the G residues that were found to be protected against methylation by DMS in vivo (Ephrussi et al., 1985). The encircled Gs are the ones whose methylation interferes with protein–DNA interaction in vitro (dotted circle indicates partial interference).

To determine the location of the binding sites within individual fragments we have used the technique of methylation interference. End-labeled DNA fragments were par-

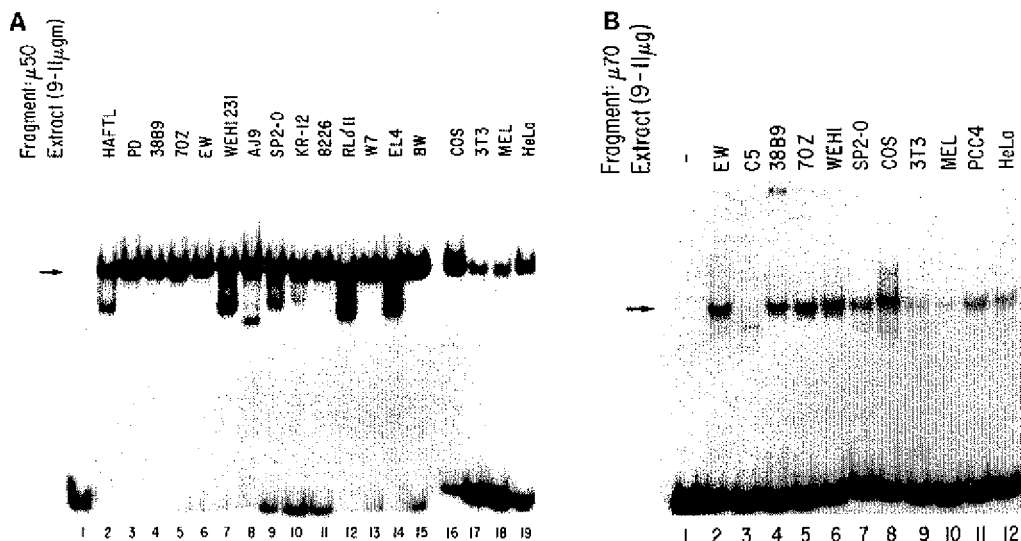


Figure 4. Analysis of  $\mu 50$  and  $\mu 70$  Binding in Extracts Generated from a Variety of Lymphoid and Nonlymphoid Cell Lines

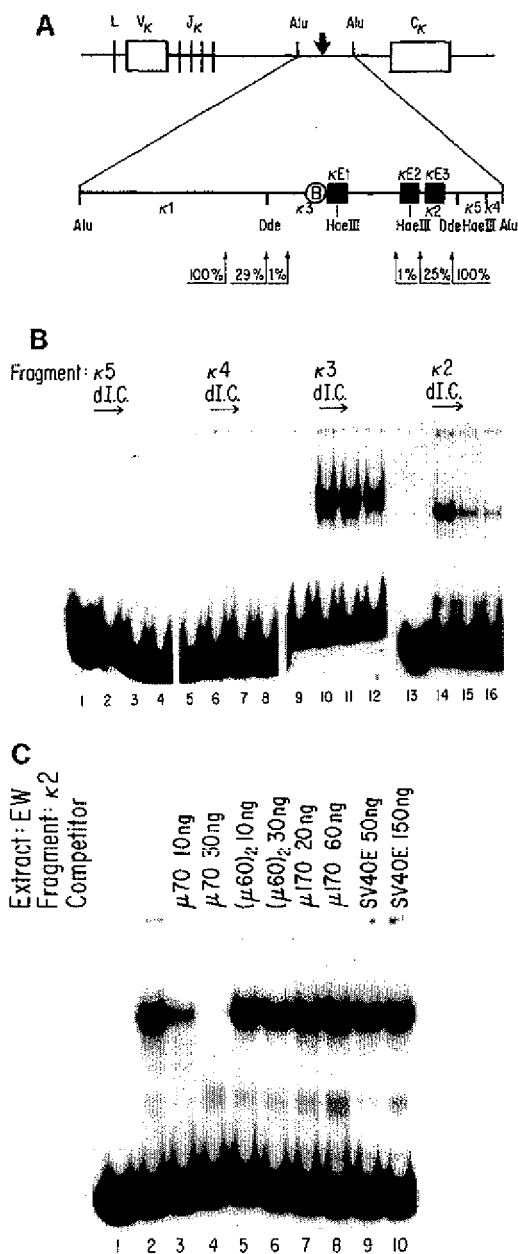
(A) End-labeled  $\mu 50$  was incubated with 9–11  $\mu\text{g}$  of protein from various cell extracts and 2.5  $\mu\text{g}$  of poly d(IC) in a 15  $\mu\text{l}$  binding reaction, followed by electrophoretic analysis. The major complex that has been characterized by competitions (Figure 2C) and methylation interference (Figure 3A) is indicated by the arrow. Lane 1, free fragment; lanes 2–19, binding analysis in various extracts. The cell lines used to derive the extracts are indicated above each lane. Briefly, HAFTL is a very early pre-B mouse cell line; PD, 3889, and 70Z are mouse pre-B cell lines; EW is a human B cell line; WEHI 231 and AJ9 are mouse B cell lines; SP2-0 is a mouse myeloma; KR12 and 8226 are human myelomas; RL 11, W7, EL4, and BW are various T cell lines; COS is a monkey cell line; 3T3 is a mouse fibroblast line; MEL is a mouse erythroleukemia line; HeLa is a human cervical carcinoma cell line.

(B) Binding of  $\mu 70$  in various extracts under the same conditions as in (A). The characterized complex is indicated by an arrow. Lane 1, free fragment; lanes 2–12, binding in various extracts.

tially methylated on guanines using DMS. Methylated DNA was then used for a binding reaction with crude extracts, and the complex was resolved from the free fragments by electrophoresis. Piperidine cleavage (Maxam and Gilbert, 1977) of eluted fragments was followed by electrophoresis through 12% polyacrylamide–urea sequencing gels. If any of the methyl groups introduced by reaction with DMS interfered with the binding of a specific protein then that molecule of DNA will be selectively missing in the complex form and subsequently in the corresponding G ladder. The method therefore allows the identification of G residues making intimate contacts with the protein. When the  $\mu 50$  DNA fragment was used in such an experiment, the free fragment generated a characteristic G ladder (Figure 3A, lanes 2 and 3) and the complex form was specifically depleted of DNA molecules carrying a methyl group at the G residue indicated by the asterisk (lane 1), which lies in the middle of the O sequence. This further implies that the NF-A protein is involved in the binding because the interaction appears to be specifically mediated by its cognate sequence. Presumably, modification of this key G residue seriously impedes the formation of a stable complex between the protein and its cognate sequence. Methylation of a second G residue (Figure 3A, lane 1, lowest of the triplet) also appeared to partially inhibit complex formation. Both of these residues have been shown to be protected against methylation by DMS *in vivo* (Ephrussi et al., 1985). On the noncoding strand, methylation of the G residue in the middle of the octamer sequence only partially inhibited complex formation (Figure

3A, lane 4, asterisk) whereas modification of any of the three A residues (marked by asterisks) abolished binding completely (lane 4). Interestingly, however, none of the other G residues protected *in vivo* in this region of the  $\mu$  enhancer appears to be involved in complex formation as measured by this methylation interference assay (Figure 3C). Therefore, if these protections *in vivo* are due to the binding of a protein, this factor is different from NF-A and is not binding to the  $\mu 50$  fragment *in vitro*.

On the  $\mu 70$  fragment several G residues were identified as being important in forming intimate contacts with the binding protein (Figure 3B). On the coding strand, bands due to three Gs (Figure 3B, asterisks) were significantly reduced in intensity in the complex as compared with the free DNA fragment (compare lanes 1 and 2); on the non-coding strand two Gs were significantly affected (compare lanes 3 and 4). In Figure 3C, open circles above the sequence indicate the residues identified by Ephrussi et al. (1985) to be protected against methylation *in vivo* whereas the encircled Gs are the ones identified by us *in vitro*. The pattern of protection and interference on the  $\mu 70$  fragment over the consensus sequence is strikingly similar *in vivo* and *in vitro*, indicating that the protein identified here may be the one that interacts with this sequence *in vivo*. Analogous to  $\mu 50$ , however, a second set of protections seen in this region *in vivo* was not observed *in vitro*. Interestingly, several G residues in the complex (Figure 3B, lane 1) appear to be more intense than the corresponding residues in free DNA (lane 2). This may mean that some of the modifications allow better interaction between the DNA



**Figure 5. Dissection and Binding Analysis of the  $\kappa$  Enhancer**  
(A) Schematic representation of the essential 475 bp Alu-Alu fragment containing the  $\kappa$  enhancer as defined by Picard and Schaffner (1984). The vertical arrow represents the approximate location of a Dnaase I hypersensitive site in the J<sub>κ</sub>-C<sub>κ</sub> intron. This enhancer was

and the specific protein presumably by subtly altering the DNA conformation.

#### Tissue Specificity of the Factors Detected

To ask whether the proteins we have identified are limited to expression only in B cells, we have screened extracts from a large number of cells (Figure 4). Complexes that comigrated with the ones generated and characterized in

further dissected by cutting with DdeI and HaeIII to generate the fragments  $\kappa$ 1 through  $\kappa$ 5, which were then used as probes in binding assays. The black boxes represent sequences homologous to the consensus sequence derived by Church et al. (1985) based on the protections against methylation by DMS observed *in vivo* on the  $\mu$  enhancer. The lowest line summarizes the results of Queen and Stafford (1984), who have carried out fine deletion mapping of the  $\kappa$  enhancer. Thus deletions from the 5' end to about 20 bp past the DdeI site cause a significant loss of enhancer function. Similarly 3' deletions extending 10–15 bp beyond the second HaeIII site cause a significant loss of enhancer function. B locates the B cell-specific protein binding site described in this paper.

(B) Binding analysis of subfragments of the  $\kappa$  enhancer. In each set of four lanes, the first shows the free fragment followed by binding analyses in the presence of increasing amount (1.2, 2.4, and 3.6  $\mu$ g) of the nonspecific competitor poly d(I,C) and a constant amount ( $\sim$ 8  $\mu$ g) of protein derived from an EW nuclear extract. Labeled fragments are as follows: lanes 1–4,  $\kappa$ 5; lanes 5–8,  $\kappa$ 4; lanes 9–12,  $\kappa$ 3; lanes 13–16,  $\kappa$ 2.

(C) Competition analysis to show that  $\kappa$ 2 interacts with the same nuclear factor that binds to  $\mu$ 70. Lane 1, free fragment; lane 2, binding of end-labeled  $\kappa$ 2 (0.2–0.3 ng, 10,000 cpm) in the absence of competitor DNA. Typical reaction contained 10  $\mu$ g protein from EW nuclear extracts and 2  $\mu$ g of poly d(I,C) in a 15  $\mu$ l volume. Lanes 3–10, binding reactions essentially as described above, but additionally containing unlabeled competitor DNA fragments derived from the  $\mu$  enhancer (refer to Figure 2A) or the SV40 enhancer (Figure 2C) in the amounts shown above each lane.

(D)  $\kappa$ 3 binds a specific factor in EW nuclear extracts. Lane 1, free fragment; lane 2, binding of end-labeled  $\kappa$ 3 (0.1–0.3 ng, 10,000 cpm) in the absence of competitor DNA. A typical reaction contained 10  $\mu$ g protein from EW nuclear extracts and 2.5  $\mu$ g of poly d(I,C) in a 15  $\mu$ l volume. Lanes 3–12, binding reactions in the presence of competitor DNAs added in the amounts shown above each lane. Refer to Figure 2C for derivations of the Kpr, KE and SV40E fragments.

the B cell line EW were observed on both the fragments ( $\mu$ 50 [Figure 4A] and  $\mu$ 70 [Figure 4B]) in all the cell lines examined. (Comparison of independent extracts indicates that estimates of the abundance of proteins in different cell lines using this assay are not meaningful.) Although the complex generated in each extract has not been further characterized, we interpret this data as indicating that both of these factors are not tissue-specific. A second

complex (having a greater mobility) was observed with the  $\mu$ 50 fragment that appears to be restricted to B and T cells only and will be described fully later (Staudt et al., unpublished results).

#### Dissection of the $\kappa$ Enhancer

An enhancer element has also been identified in the major intron of the  $\kappa$  light chain gene. Picard and Schaffner (1984) showed that the enhancement activity can be localized to a 500 bp AluI-AluI fragment, and Queen and Stafford (1984) have shown that deletion of the 5' AluI-DdeI fragment has a minimal effect on enhancer activity, restricting the enhancer to 275 bp from DdeI to the 3' AluI site (Figure 5A; the black boxes represent sequences identified by Church et al. [1985] as homologous to the series of E domains detected in the  $\mu$  enhancer). Fragments were generated by cutting with DdeI and HaeIII (see Figure 5A) and assayed for binding in the presence of increasing amounts of poly d(IC) as a nonspecific competitor. Fragments  $\kappa$ 4 and  $\kappa$ 5 appeared negative (Figure 5B, lanes 1-8), while  $\kappa$ 3 and  $\kappa$ 2 formed complexes (lanes 10-12 and 14-16).  $\kappa$ 1 is too large a fragment to be reliably assayed and has not been further dissected yet. Preliminary results show that the internal undesigned HaeIII fragment does not contain any specific binding sites either. The competition pattern for  $\kappa$ 2 was strikingly similar to what had been observed earlier with the  $\mu$ 70 fragment: relatively large amounts of  $\mu$ 400, the Moloney leukemia virus enhancer, the SV40 enhancer, or the  $\kappa$  promoter (containing the O sequence) did not compete for binding, while  $\mu$ 300 and the  $\kappa$  enhancer did (data not shown). Because  $\kappa$ 2 contains a putative E box identified by sequence comparison (as does  $\mu$ 70), we competed away its binding with smaller fragments from  $\mu$ 300 (Figure 5C). The complex is specifically competed away by the addition of unlabeled  $\mu$ 70 during the incubation (Figure 5C, compare lanes 3 and 4 with lane 2), but not by the addition of ( $\mu$ 60)<sub>2</sub> (lanes 5 and 6),  $\mu$ 170 (lanes 7 and 8), or the SV40 enhancer (lanes 9 and 10). Furthermore, the protein that binds to this sequence cofractionates with NF- $\mu$ E3, the  $\mu$ 70 binding activity, through two sequential chromatographic steps (heparin agarose and DEAE-Sephadex) (data not shown). We conclude that the same sequence-specific protein (NF- $\mu$ E3) binds to both fragment  $\mu$ 70 and fragment  $\kappa$ 2; therefore, at least one common protein interacts with both the  $\mu$  and the  $\kappa$  enhancers.

The  $\kappa$ 3 complex (Figure 5D, arrowhead) failed to be competed away by  $\mu$ 300 (lanes 3 and 4),  $\mu$ 400 (lanes 5 and 6), or a  $\kappa$  promoter-containing fragment (lanes 7 and 8). The complex, however, was specifically competed away by both the complete  $\kappa$  enhancer (Figure 5D, lanes 9 and 10) and the SV40 enhancer (lanes 11 and 12). The band below the major  $\kappa$ 3 complex was seen at variable intensities in different experiments and failed to compete even with the complete  $\kappa$  enhancer in this experiment and has not been further investigated. The observation that the SV40 enhancer specifically competes for binding of this factor suggests that an identical stretch of 11 nucleotides (GGGGACTTTCC) shared with the SV40 enhancer may be responsible for the binding.

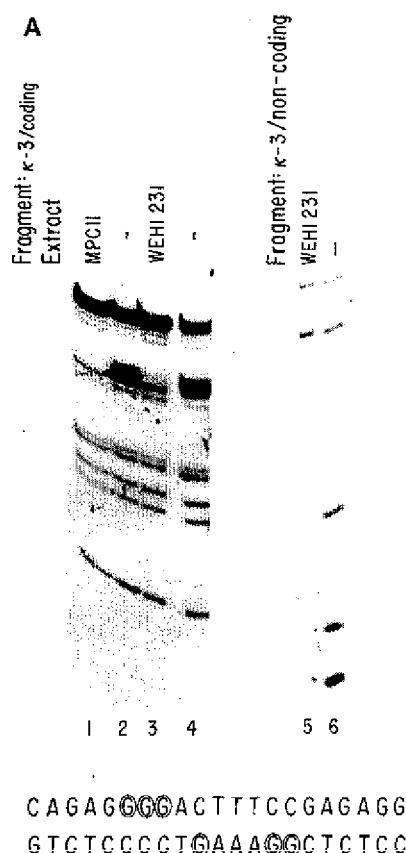


Figure 6. Methylation Interference Analysis of the Nucleoprotein Complex Generated on  $\kappa$ 3

(A) Preparative binding reactions were carried out with partially methylated  $\kappa$ 3 end-labeled at the DdeI site in two different extracts: MPC11 (mouse myeloma) and WEHI 231 (mouse B cell line). The complex and free fragment bands were eluted from a low ionic strength polyacrylamide gel, treated with piperidine, and analyzed by electrophoresis through a 12% sequencing gel. Lanes 1 and 3, G ladder corresponding to the nucleoprotein complex bands generated in MPC11 extracts and WEHI 231 extracts, respectively. Lanes 2 and 4, G ladder corresponding to the free fragment isolated after binding in MPC11 and WEHI 231 extracts, respectively. Lanes 5 and 6, analysis of the G residues on the noncoding strand in the nucleoprotein complex band (lane 5) and the free fragment (lane 6) following preparative binding and low ionic strength gel electrophoresis. G residues whose methylation interferes with nucleoprotein complex formation are indicated by the asterisks.

(B) Summary of the methylation interference (in vitro) experiments used to define the B site within the  $\kappa$  enhancer. The relevant region of the  $\kappa$ 3 fragment is shown with the coding strand on top. The encircled Gs are the ones whose methylation interferes with protein DNA interaction.

We have localized the binding site of this factor on the  $\kappa$ 3 fragment by carrying out methylation interference experiments. In two different extracts, methylation at three of a stretch of four G residues on the coding strand within the sequence shared with SV40 completely abolished binding (Figure 6A, compare the complexed fragments in lanes 1 and 3 with the free fragments in lanes 2 and 4; asterisks indicate the position of Gs whose methylation seriously interferes with binding). On the noncoding strand, methyla-

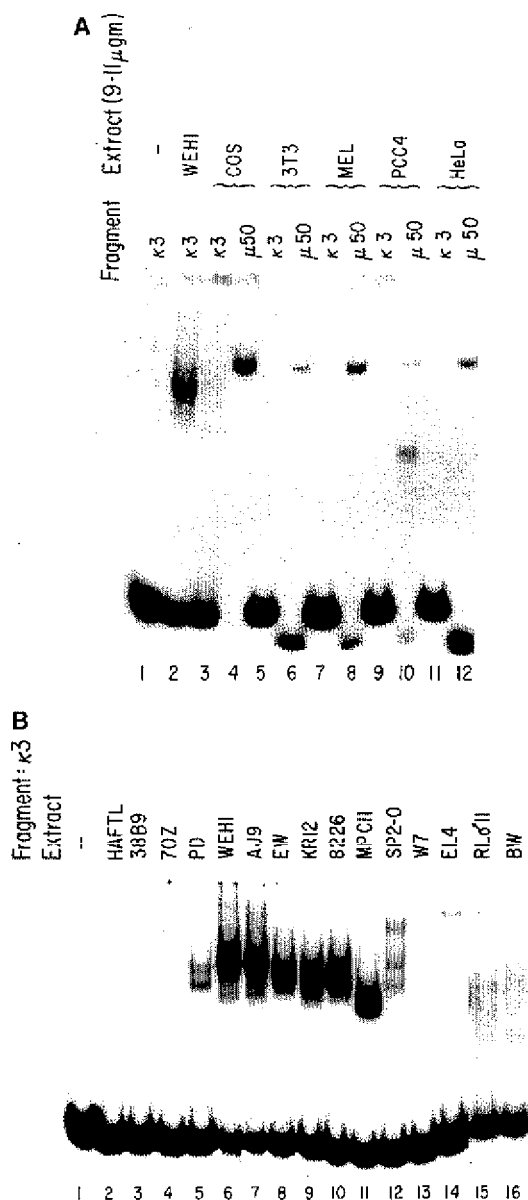


Figure 7. Analysis of  $\kappa 3$  Binding in a Variety of Lymphoid and Nontymphoid Cell Extracts

(A)  $\kappa 3$  binding reactions carried out in five nontymphoid lines. Lane 1, free fragment; lane 2, binding in a cell line positive for this factor (WEHI 231). A typical reaction had 0.1–0.3 ng (10,000 cpm) of labeled fragment together with 2.5  $\mu$ g of poly d(I/C) and 9–11  $\mu$ g of nuclear extract. Odd numbered lanes 3–11, binding reactions in the same conditions as above in a variety of extracts generated from nontymphoid lines as noted above each lane (refer to Experimental Procedures for a description of these lines). Even numbered lanes 4–12, binding of  $\mu 50$  (which detects a ubiquitous factor) under the same conditions, serving as a positive control for each extract.

(B)  $\kappa 3$  binding reactions in lymphoid cell extracts. Lane 1, free fragment. Binding reactions were performed, as detailed above, in extracts derived from pre-B cell lines, (HAFTL, 38B9, 70Z, and PD) (lanes 2–5); B cell lines (WEHI 231, AJ9, and EW) (lanes 6–8); plasma cell lines (KR12, 8226, SP2-0, and MPC11) (lanes 9–12); and T cell lines (lanes 13–16). Note that the mobility of the complex formed differs slightly between extracts derived from mouse cells (e.g., AJ9, WEHI 231) or hu-

man cells (EW, KR12, and 8226). The significantly altered mobility in the MPC11 extracts is probably due to proteolysis, since many other binding sites also show higher mobility complexes in this extract.

tion of three G residues (Figure 6A, lane 5, asterisks) inhibited complex formation. Thus the binding site was (shown in Figure 6B; circled G residues are those determined by methylation interference experiments to be important for complex formation) localized toward one end of the  $\kappa 3$  fragment (Figure 5A, represented by B). This result also served to explain the specific competition observed earlier with the SV40 enhancer. Interestingly, deletion mapping of the  $\kappa$  enhancer was shown that sequences within the  $\kappa 3$  fragment are extremely important for enhancer function (Queen and Stafford, 1984).

The tissue range of this factor was examined by carrying out binding analysis with  $\kappa 3$  using extracts from a variety of cell lines. Nucleoprotein complex formation with  $\kappa 3$  was detected in a mouse B cell line (Figure 7A, lane 2) but not in 5 other non-B cell lines (odd numbered lanes from 3–11). Even numbered lanes in Figure 7A show that the ubiquitous factor detected by  $\mu 50$  is present in all of these cell lines and serves as a positive control for the experiment. The factor therefore appears to be restricted in expression to B lymphoid cells. We then examined extracts made from cells at various stages of B cell differentiation (Figure 7B). Interestingly,  $\kappa 3$  binding protein was detected in the pre-B cell line PD (Figure 7B, lane 5), which spontaneously rearranges its  $\kappa$  genes (Lewis et al., 1982); two mouse B cell lines (WEHI 231 and AJ9, lanes 6 and 7); one human B cell line (EW, lane 8); two mouse myeloma cell lines (MPC11, SP2-0, lanes 11 and 12); and two human myelomas (KR12 and 8226, lanes 9 and 10). However, it was not apparent in a very early pre-B cell line (HAFTL, Figure 7B, lane 2) and two standard mouse pre-B cell lines (38B9 and 70Z, lanes 3 and 4). Thus this factor appears to be not only tissue-specific and limited to cells of the B lymphoid lineage, but also stage-specific within that lineage. For these reasons, we refer to the binding site for this factor as the B site and refer to the factor as NF- $\kappa$ B.

In the series of extracts examined, the presence of the NF- $\kappa$ B factor is strikingly correlated with  $\kappa$  gene expression, but there is one apparent discrepancy concerning its stage specificity. Cell line PD, which was derived by Abelson murine leukemia virus transformation of adult bone marrow cells (Rosenberg and Baltimore, 1976) and undergoes  $\kappa$  light chain rearrangement in culture, had the factor. Cell line 70Z, which is apparently further along the B cell differentiation pathway relative to PD, having already completed rearranging its  $\kappa$  light chain genes (Maki et al., 1980), had no detectable factor. We believe that these may not be contradictory because 70Z cells do not actively transcribe the  $\kappa$  locus and do not have the DNAase I hypersensitive site (Parslow and Granner, 1982) in the  $J_{\kappa}$ - $C_{\kappa}$  intron that has been correlated with  $\kappa$  gene expression. For PD, however, the  $\kappa$  enhancer is active after transfection (Speck and Baltimore, unpublished results); and the DNAase I hypersensitive site associated with the  $\kappa$  enhancer can be detected (Sen and Baltimore, unpublished results).

Table 1. Summary of Immunoglobulin Enhancer-Binding Factors

Factor	Binding Site(s)	Tissue Distribution
NF-A	Octamer sequence (ATTGTCAT) in V(H), V(L) promoters and $\mu$ enhancer	Ubiquitous (B cell specific component)
NF- $\mu$ E3	E3 site in $\mu$ enhancer	Ubiquitous
NF- $\mu$ E1	E1 site in $\mu$ enhancer	Ubiquitous
NF- $\kappa$ B	B site in $\kappa$ enhancer	$\kappa$ -Producing B cells only

E1, E2, E3, etc., refer to the E homology identified by Ephrussi et al. (1985). NF- $\mu$ E1 has been identified by Weinberger et al. (1986).

## Discussion

We have detected interaction of multiple factors (summarized in Table 1) with Ig  $\mu$  and  $\kappa$  enhancer sequences using an electrophoretic mobility shift assay. Within the 300 bp PstI-EcoRI fragment of the  $\mu$  enhancer, two sites have been localized. One is an octamer (O) sequence (ATTGTCAT) that is also conserved upstream of all heavy and  $\kappa$  chain variable region genes and appears to bind the ubiquitous NF-A factor. The second sequence coincides with one of the motifs ( $\mu$ E3) shown by Ephrussi et al. (1985) and by Church et al. (1985) to be bound to a factor in B cells. Both of the factors were detected in a broad range of cells and therefore did not appear tissue-specific. There appears also to be a tissue-specific factor that can bind to the O sequence (Staudt et al., unpublished results). The  $\mu$ E1 and  $\mu$ E4 sequences that have close homology to the  $\mu$ E3 sequence competed poorly, if at all, for binding to the  $\mu$ E3 sequence, implying that the sequence specificity of the  $\mu$ E3-binding protein is quite exquisite. A different protein has been shown to bind to  $\mu$ E1 (Weinberger et al., 1986).

Our dissection of the  $\kappa$  enhancer has revealed two binding sites. One of these,  $\kappa$ E3, corresponds closely in sequence to one of the  $\mu$  sites,  $\mu$ E3 (see Figure 8), and its binding protein appears to be NF- $\mu$ E3, the factor that binds to  $\mu$ E3. Thus there is at least one protein that interacts with both the  $\mu$  enhancer and the  $\kappa$  enhancer. The second site, B, is the most unique one we have found because NF- $\kappa$ B, its binding protein, is restricted in appearance to cells that ordinarily express  $\kappa$  chains. It is not present in the  $\mu$  enhancer, but is found in the SV40 enhancer.

Although all of the data presented here involved crude nuclear extracts as the source of protein, preliminary results of chromatographic fractionation are consistent with the proposal that at least three separable factors interact with the immunoglobulin enhancers.

The enhancers are defined partly by their ability to enhance transcription when present in either orientation relative to a promoter. It might therefore be thought that their binding sites should have dyad symmetry. The E series of sequences (those suggested by Ephrussi et al. [1985] to be homologous) do contain an element of dyad symmetry.



Figure 8. Comparison of E Domains from the  $\mu$  Enhancer and the  $\kappa$  Enhancer

$\mu$ E1- $\mu$ E4 were defined by Ephrussi and Church in the  $\mu$  enhancer on the basis of methylation protection experiments in vivo (Ephrussi et al., 1985).  $\kappa$ E1- $\kappa$ E3 were identified within the  $\kappa$  enhancer as being homologous to the consensus sequence derived by comparing  $\mu$ E1- $\mu$ E4 (Church et al., 1985) (see Figure 5A). The arrows over the sequence point out a mini dyad axis of symmetry within each domain. (These also include in every case the most conserved residues in the consensus sequence [Church et al., 1985].) In the fragments used by us to dissect the enhancers,  $\mu$ E3 is completely present within the  $\mu$ 70 fragment,  $\mu$ E4 within the  $\mu$ 50 and  $\mu$ 170 fragments, and  $\kappa$ E3 within the  $\kappa$ 2 fragment.

As illustrated in Figure 8, there is a motif of CA/TG in all of these sequences with a separation of two or three bases. One of the next outer two bases also has a symmetric counterpart giving a dyad of three out of four bases in each sequence. The two sequences that appear to bind the same factor ( $\mu$ E3 and  $\kappa$ E2) also have identical internal two base spacers of TG. Of those that do not compete for binding with  $\mu$ E3 and  $\kappa$ E2,  $\mu$ E1 has a three base spacer and  $\mu$ E4 has a spacer of GG. Another related sequence ( $\kappa$ E2) within the  $\kappa$  enhancer also has a GG dinucleotide within its inverted repeat but has yet to be analyzed. We have been unable to detect any binding to  $\mu$ E4 thus far, but  $\mu$ E1 appears to have a specific binding factor (Weinberger et al., 1986). The inability of such closely homologous sequences (particularly  $\mu$ E4) to compete for binding to  $\mu$ E3 suggests that these nuclear binding proteins may have restriction enzyme-like specificity in their binding. Perhaps some of the enhancer-binding proteins belong to families of related proteins with slightly different binding specificities.

Two of the identified binding sites have no homology either to the E series, or to each other. One, the O sequence from the  $\mu$  enhancer (ATTGTCAT), is bound by NF-A, a non-tissue-specific factor, but also is bound by a factor found only in cells of the B lymphoid series (Staudt et al., unpublished results). The other, the B sequence from the  $\kappa$  enhancer (found around the sequence GGGGACTTTC), binds to NF- $\kappa$ B, a factor specific to cells that express  $\kappa$  chains. Ignoring the nonspecific factor that binds to the O sequence, it might appear that each enhancer has E-related elements that bind to nonspecific factors and unique elements that bind to specific factors and that could be responsible for the tissue specificity of the individual enhancers. As discussed elsewhere (Singh et al., 1986), the existence of a nonspecific factor that binds to the O sequence might relate to the use of this site for

regulating transcription by promoters such as those for the U1 and U2 RNAs.

There is an apparent discrepancy between the *in vivo* binding data (Mercola et al., 1985; Ephrussi et al., 1985) and the *in vitro* data we have accumulated. Ephrussi et al. (1985) and Church et al. (1985) find that the E sites and the O site are protected against methylation in B cells but not in fibroblasts. We find that NF- $\mu$ E3 and NF-A are present in fibroblastic and other nonlymphoid cells. It would therefore appear that the mere presence of a factor is not sufficient for it to bind in such a way as to generate protection of the G residues at the site *in vivo*; in all likelihood, the protein is not bound to a site except in specific cells. This implies that for binding to occur, a given segment of DNA may have to be "activated," a process that may involve making chromatin accessible to the binding factors. It is possible that tissue-specific binding proteins play the role of activation and therefore open the DNA to interaction with nonspecific transcription enhancing proteins. *In vitro*, where naked DNA is used for assay, such interactions would not be evident and the factors would all appear to be equivalent DNA binding proteins.

Although one might expect that at least some of the sequences to which factors bind in the tissue-specific Ig enhancers would be unique to these structures, most of the sequences have close relatives in other regulatory elements. The two sequences most likely to be involved in tissue specificity—the O sequence and the B sequence—are both found in the SV40 enhancer, and that viral sequence will compete for binding of factors to O and B *in vitro*. Transcriptional potentiation by the SV40 enhancer can be competed away by the  $\mu$  enhancer both *in vivo* and *in vitro*, suggesting that a common factor may interact with both of these sequences (Mercola et al., 1985; Sassone-Corsi et al., 1985). A possible explanation for this could be that when the SV40 enhancer is activated in a non-B cell, it uses some other sequences or binding sites for its activity, whereas when it is activated in a B cell, it uses, for instance, its homolog of the NF- $\kappa$ B binding site. In this model, the SV40 enhancer could be a mosaic of different sequence motifs recognizing a number of different factors, only a subset of which is needed in any one cell to generate function.

In previous studies using the mobility shift assay, we have determined the site of binding using a variant of the DNAase I footprinting method (Singh et al., 1986). In the present study we have used a methylation interference assay both because it allows a higher resolution analysis of the binding site and because we found that many complexes cannot be assayed by the footprint method. For those complexes that do not yield a footprint, there appears to be too rapid an equilibration between complexed and free DNA to allow complexes to be treated with DNAase and then resolved by electrophoresis. For instance, the half-life of the nucleoprotein complex with the  $\mu$ 70 fragment is less than a minute (Sen, unpublished observation). In the methylation interference protocol, DNA that is methylated will not rebind eluted proteins, and therefore the bound complexes never contain DNA methylated at a residue critical to the binding reaction.

## Experimental Procedures

### Extracts and Cell Lines

Nuclear extracts were made from the following tissue culture cell lines exactly according to the protocol of Dignam et al. (1983) and usually contained 6–12 mg/ml of protein: HAFTL, Harvey sarcoma virus transformant (Pierce and Aaronson, 1982), which presumably represents an early stage in B cell differentiation because it is still in the process of carrying out D<sub>H</sub>→J<sub>H</sub> rearrangements at the immunoglobulin heavy chain locus (Desiderio and Baltimore, unpublished results); 38B9 and PD, Abelson murine leukemia virus transformants, which are pre-B-like because they either contain a rearranged (VDJ) heavy chain locus (PD, Lewis et al., 1982) or are in the process of assembling their heavy chain genes (38B9, Yancopoulos et al., 1984); 70Z, mouse pre-B cell line; WEHI 231 and AJ8, mouse B cell lines containing functionally rearranged heavy and light chain genes; EW 36, human EBV-negative Burkitt lymphoma; KR12 and 8226, human myelomas (gift from Dr. C. M. Croce); SP2-0 and MPC 11, mouse myelomas; BW5147, W7, EL4, and RL $\sigma$ 11, mouse T cell lines; COS, monkey cell line; 3T3, mouse fibroblast cell line; MEL, mouse erythroleukemia cell line; PCC4, mouse embryonic carcinoma line; HeLa, human cervical carcinoma cell line.

Plasmids were constructed as follows. The 300 bp PvuII–EcoRI fragment of the  $\mu$  enhancer was digested with AluI, and the fragments were subcloned into pUC13 cut with SmaI to yield  $\mu$ 70 (containing the 70 bp AluI–AluI insert) and  $\mu$ 170 (containing the 170 bp AluI–AluI insert). See Figure 2A for a restriction map of the relevant region.

Competitor DNA corresponding to the various  $\mu$ E boxes were prepared as follows:  $\mu$ E1, by BamHI–PvuII digestion of  $\mu$ Ec (a plasmid containing the 220 bp HinfI–HinfI fragment of the  $\mu$  enhancer, subcloned into SmaI-cut pUC13, which was a gift from Dr. J. Weinberger);  $\mu$ E3, by EcoRI and BamHI digestion of  $\mu$ 70;  $\mu$ E4, by HinfI–DdeI digestion of the insert obtained by cleaving  $\mu$ 170 with EcoRI and BamHI.

The 475 bp AluI–AluI fragment containing the  $\kappa$  enhancer ( $\kappa$ E) was subcloned into pUC13 cut with SmaI. Competitor DNA was prepared by cutting at flanking sites within the polylinker. DdeI and HaeIII were used to generate the various smaller fragments as shown in Figure 5A. The  $\kappa$  promoter ( $\kappa$ pr) was obtained from a plasmid that contained approximately 300 bp (spanning positions –35 to –330 relative to the cap site of the MOFC 41  $\kappa$  gene) of an SfaNI fragment that was subcloned into SmaI-cut pSP64 (a gift of Dr. N. E. Speck). Large competitor fragments (greater than 150 bp) were isolated from low melting point agarose gels by four extractions with phenol and one extraction with chloroform, followed by precipitation with ethanol. Smaller competitor fragments were isolated from 8%–12% native polyacrylamide gels by soaking the minced gel slice in elution buffer containing 500 mM ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, and 0.1% SDS. After a 6–8 hr incubation at 37°C, the supernatant was extracted once with phenol and once with chloroform and the DNA was precipitated by adding 2.5–3 volumes of ethanol. Competitor DNA was quantified by comparison to standard weights of DNA either after electrophoresis through agarose or after spotting onto an agarose plate. Radioactive probe was obtained by end-labeling dephosphorylated DNA with [ $\gamma$ -<sup>32</sup>P]ATP in the presence of polynucleotide kinase (Boehringer Mannheim Biochemicals). Typical specific activities ranged from 30,000–70,000 cpm/ng of DNA.

### Gel Binding Analysis

Binding reactions were carried out in 10 mM Tris (pH 7.5), 40 mM NaCl, 1 mM EDTA, 1 mM  $\beta$ -mercaptoethanol, and 4% glycerol for 20–30 min at room temperature. Poly d(IG) was added as a nonspecific carrier, and a typical reaction contained 10,000 cpm (0.2–0.5 ng) of end-labeled DNA with 9–11  $\mu$ g of extract (which was added last). Following binding, the mixture was electrophoresed through a native 4% polyacrylamide gel (acrylamide: bisacrylamide ratio 30:1) containing 6.7 mM Tris-HCl (pH 7.5), 3.3 mM sodium acetate, and 1 mM EDTA. The gel was pre-electrophoresed for 2 hr at 11 V/cm. Electrophoresis was carried out at the same voltage for 2 hr at room temperature with buffer recirculation. The gel was then dried and autoradiographed with a screen at –70°C. For competition experiments the conditions were exactly as above, except that specific and nonspecific competitor DNAs were included in the mixture (in amounts as detailed in the figure legends) prior to addition of the protein.

### Methylation Interference Experiments

End-labeled DNA fragments were partially methylated at the guanine residues, as detailed by Maxam and Gilbert (1977) with the following modification. The reaction was quenched with 1.5 M sodium acetate (pH 7.0), 1 M  $\beta$ -mercaptoethanol, and 100  $\mu$ g/ml of poly d(I/C). Methylated DNA was precipitated twice, rinsed with 70% ethanol, dried, and taken up in TE (10 mM Tris [pH 8.0] and 1 mM EDTA). For a typical preparative binding reaction, the usual conditions were scaled up 5 to 10 fold. Binding and gel electrophoresis were as above. After electrophoresis the gel was wrapped with Saran wrap and exposed wet for 4–6 hr at room temperature. The complex and free fragment bands were then excised and electroeluted for 1–2 hr (RNA was added to prevent adsorption of labeled DNA to the membrane) to recover the DNA. Prior to ethanol precipitation, the solution was extracted sequentially with phenol and chloroform. The pellet was rinsed thoroughly with cold 70% ethanol, dried, then redissolved in 100  $\mu$ l of 1 M piperidine. Base cleavage reactions were carried out for 45 min at 90°C followed by removal of the piperidine by lyophilization. After two additional rounds of lyophilization from water, the products were analyzed by separation by electrophoresis through a 12% polyacrylamide gel in the presence of 8 M urea followed by autoradiography at –70°C with a screen.

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the thymus of  $TL^b$  mice. But it is clear that most of these cells are not eliminated even though they interact with products of the  $TL^b$  haplotype in the thymus. As a result of this interaction, KN6 TCR-positive cells increase in size, down-modulate their TCR and probably express IL-2-receptor  $\beta$ -chains, which would confer on them the ability to respond to IL-2 alone<sup>14</sup>. This effect of the  $TL^b$  product does not reflect an essential positive selection step, because KN6 TCR-positive cells do survive in, and are exported from, the thymus of  $TL^d$  transgenic mice. Whereas KN6 TCR-positive cells from  $TL^d$  thymi respond to  $TL^b$  stimulator cells with IL-2 production and proliferation, KN6 TCR-positive cells from  $TL^b$  thymi lose the capacity to produce IL-2 and, as a result, respond to  $TL^b$  stimulator cells only in the presence of exogenous IL-2. Therefore the response of KN6 TCR-positive cells from  $TL^b$  mice to  $TL^b$  stimulator cells is dependent on helper cells that supply IL-2. The lack of such helper cells in  $TL^b$  mice could explain the lack of destructive

autoimmune responses in  $TL^b$  transgenic mice and the lower number of transgenic TCR-positive cells in the periphery of  $TL^b$  mice versus  $TL^d$  mice. The unresponsive state of the TG-positive cells that is generated in the thymus of the transgenic mice used in the study described here is reminiscent of the state of clonal energy that can be induced in peripheral  $\alpha\beta$  T cells<sup>15-18</sup>, as well as in the thymocytes of  $P \rightarrow F_1$  chimaeras<sup>19</sup>.

Our study shows that certain T cells may become dependent on helper cells if they encounter self-antigen in the thymus as well as in the periphery. Like self antigen-specific B cells, self antigen-specific T cells are not necessarily harmful to the host. In fact they could have beneficial functions provided that helper cells are absent, which would facilitate their proliferation and differentiation to potentially harmful effector cells in response to host components. □

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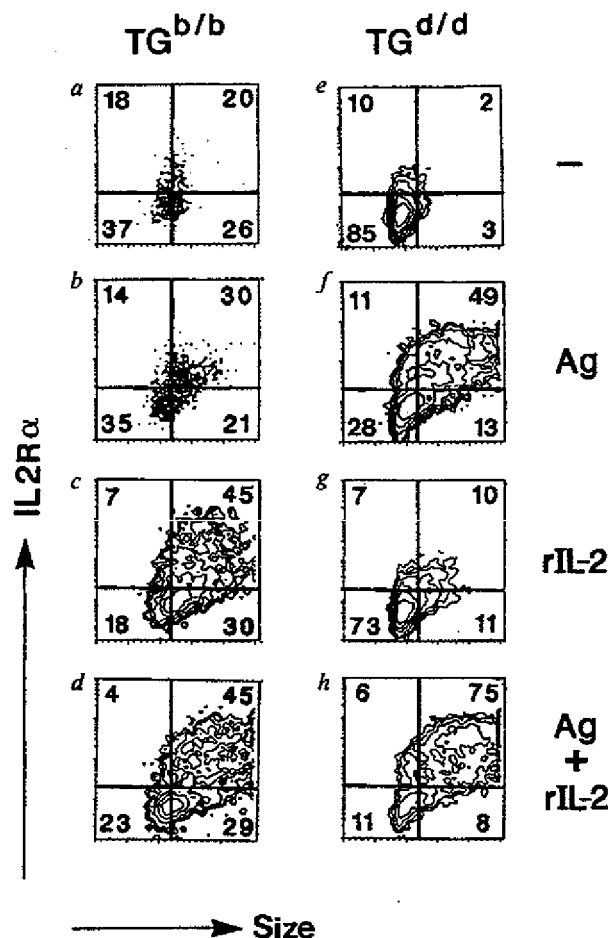
## A second B cell-specific enhancer 3' of the immunoglobulin heavy-chain locus

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THE expression of immunoglobulin heavy-chain (IgH) genes is generally thought to be regulated by the combination of the  $VH$  promoter with the enhancer element which is located in the  $JH-CH$  intron<sup>1-4</sup>. This is probably an oversimplification: there are cell lines that transcribe IgH genes despite the deletion of the intron-enhancer<sup>5-8</sup>. These findings could imply that other enhancer element(s) exist in the IgH locus<sup>9-11</sup>. Here we show that a strong B-cell-specific enhancer is indeed located at the 3'-end of the rat IgH locus, 25 kilobases downstream of  $C\alpha$ . This enhancer should be retained downstream of all rearranged IgH genes, regardless of the  $VH$  or  $CH$  segment used. Taken together with analogous findings for the mouse  $\kappa$  locus<sup>12</sup>, the results prompt a re-evaluation

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**FIG. 3** Blastogenesis and IL-2 receptor  $\alpha$ -chain (IL-2R $\alpha$ ) expression of cultured DN thymocytes from KN6 TG mice. DN thymocytes from transgenic mice of  $TL^b$  ( $TG^{b/b}$ ) or  $TL^d$  ( $TG^{d/d}$ ) haplotypes were cultured in culture medium alone (a, e), with irradiated  $TL^b$  peritoneal cells (b, f), with rIL-2 alone (c, g), or with rIL-2 and  $TL^b$  stimulator cells (d, h). After 2 days of culture, cells were stained with 8D6 (anti-V $\gamma$ 4V85) and PC61 (anti-IL-2R $\alpha$ ) monoclonal antibodies. Shown are the dot-plot histograms (IL-2R $\alpha$  fluorescence intensity ( $\log_{10}$  scale) on the vertical axis, and forward scatter (FSC; linear scale) on the horizontal axis of gated 8D6<sup>+</sup> cells, and the percentage of cells in each quadrant.

**METHODS.** Cultured cells were incubated with PC61 monoclonal antibody (ATCC No. T1B222) followed by FITC-coupled purified goat anti-rat IgG antiserum (CALTAG, San Francisco). After a third incubation in 10% normal rat serum, cells were stained with biotin-coupled 8D6 monoclonal antibody followed by streptavidin-phycoerythrin. Flow cytometry was performed according to procedures described in Fig. 1 legend.

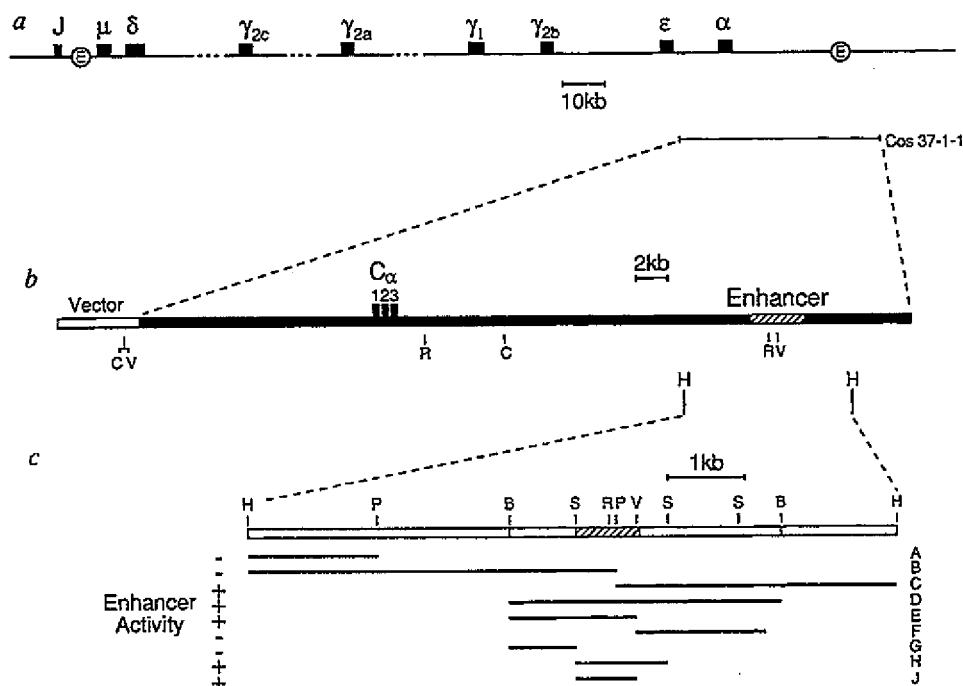


FIG. 1 a The rat IgH locus.  $\oplus$  denotes the intron and 3' enhancers. The interrupted line linking  $\gamma_{2c}$  and  $\gamma_{2a}$  indicates that the genes have not been physically linked; the gene order derives from analysis of gene deletions in hybridomas<sup>14</sup>; the rest of the rat IgH genes have been linked on overlapping cosmids<sup>13</sup>. b, Map of cosmid 37-1-1. c, Map of the HindIII enhancer fragment and the enhancer activity of subfragments. Assays were performed as described in the legend to Fig. 2. Restriction enzyme cleavage sites: B, *Bgl*II; C, *Clat*; H, *Hind*III; P, *Pst*I; R, *Eco*RI; S, *Stu*I; Sm, *Sma*I and V, *Eco*RV. The single *Pst*I site indicated in the shaded enhancer fragment is a doublet.

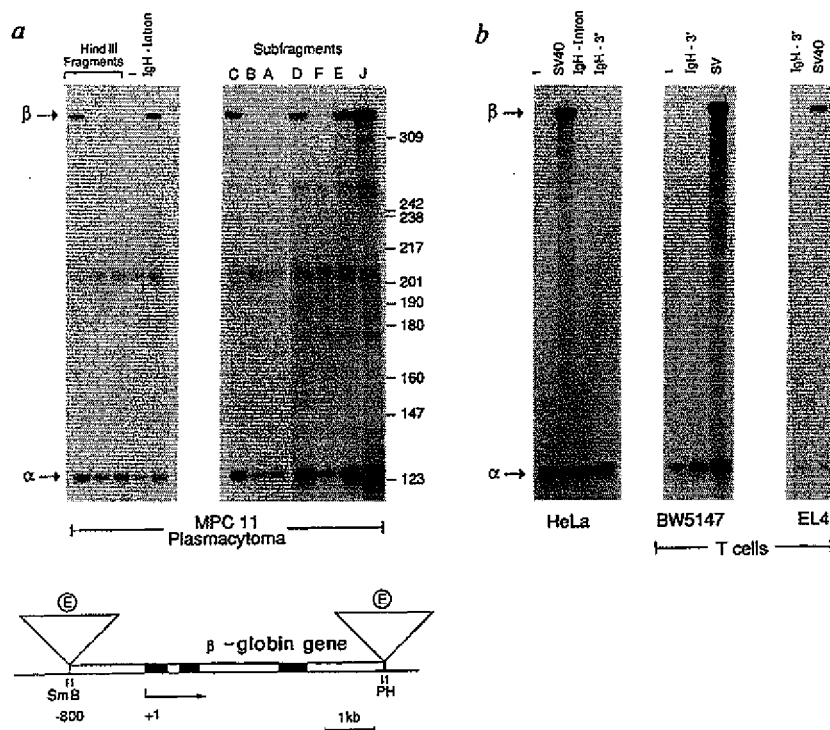
of the mechanism of regulation of immunoglobulin gene transcription. Furthermore, unlike the intron-enhancer, the IgH 3' enhancer would become linked to a c-myc that rearranges into an IgH switch region. The IgH 3' enhancer could therefore play a part in the activation of the translocated c-myc genes in rat immunocytomas, mouse plasmacytomas and Burkitt lymphomas.

In screening for an additional enhancer in the IgH locus, we focused our attention on sequences 3' of  $C_\alpha$ , because this region is likely to be maintained in all rearranged IgH genes regardless of the particular VH or CH gene used. We used cosmids that covered >30 kilobases (kb) of the 3' end of the rat IgH locus. The locus of rat (Fig. 1a) has striking homology to that of the

mouse both in overall organization and in sequence. For example, the rat intron-enhancer and many of the individual CH genes show >90% homology to their mouse counterparts<sup>13,14</sup>.

To test for enhancer activity, we exploited the fact that the human  $\beta$ -globin gene in plasmid p $\beta$ 800 (Fig. 2a) is only a weak transcription unit in transfected cells unless it is provided with an exogenous enhancer element. We scanned cosmid 37-1-1 for enhancer elements because this cosmid spans  $C_\alpha$  and the region 3' of it (Fig. 1a). Random *Hind*III fragments of Cos 37-1-1 were inserted downstream of the  $\beta$ -globin gene in plasmid p $\beta$ 800. The DNA from the resulting constructs was transfected into the

FIG. 2 a Location of the rat IgH 3' enhancer. Ribonuclease protection assay measurements of human  $\beta$ - and  $\alpha$ -globin mRNAs in transfected mouse MPC11 plasmacytoma cells. Cells were transfected with derivatives of plasmids p $\beta$ 800 (see diagram) or p $\beta$ 128, along with a reference plasmid that included the human  $\alpha_2$ -globin gene. Bands corresponding to correctly initiated  $\beta$ - and  $\alpha$ -globin transcripts are indicated. Plasmid p $\beta$ 800 (gift from K. Weston) consists of the human  $\beta$ -globin gene with 800 nucleotides (nt) of 5' flanking sequence cloned between the *Hinc*II and *Pst*I sites of plasmid pUC12; plasmid p $\beta$ 128 includes 128 nt of 5' flanking sequence. The derivatives of plasmid p $\beta$ 800 include *Hind*III (left panel) or *Pst*I fragments (fragments A, B and C) cloned downstream of  $\beta$ -globin gene, whereas the other fragments were cloned upstream. The mouse IgH-intron enhancer (IgH-Intron; 1-kb *Xba*I fragment) served as a positive control. b, Cell-type specificity of the IgH 3' enhancer (IgH-3'; *Stu*I-*Eco*RV fragment). Parallel assays were performed in HeLa cells and in mouse thymoma cell lines EL4 and BW5147. The SV40 enhancer served as a positive control. Transfection of MPC11 and HeLa cells by calcium phosphate co-precipitation, transfection of T-cell lymphomas by use of DEAE-dextran, and measurements of RNA levels, were carried out as previously described<sup>4,12</sup>.



mouse plasmacytoma cell line MPC11 by calcium phosphate co-precipitation along with a human  $\alpha 2$ -globin plasmid that served as an internal reference. The amount of  $\beta$ - and  $\alpha$ -globin messenger RNAs produced by the transfected cells was then measured in ribonuclease protection assays. One of the *Hind*III fragments caused a considerable stimulation of  $\beta$ -globin transcription and gave rise to mRNA levels comparable to those achieved with the IgH intron-enhancer (Fig. 2a). Mapping of this *Hind*III fragment revealed that it contained sites for *Eco*RI and *Eco*RV, which allowed us to identify its position relative to the  $\alpha$  gene (Fig. 1b). Further fragmentation of the cosmid and of clones derived from it demonstrated that enhancer activity was in a 0.7-kb *Stu*I-*Eco*RV fragment located 25 kb downstream of the  $\alpha$  exons (Fig. 1c). This IgH 3' enhancer has the classic properties of a transcriptional enhancer element in that it is active in both orientations and functions both upstream and downstream of the test gene. (The *Hind*III fragments and *Pst*I fragments A, B and C were assayed downstream of the  $\beta$ -globin gene, whereas fragments D-J were assayed upstream. Similarly, fragments H and J were assayed in opposite orientations to each other). To test whether the 3' enhancer possessed ubiquitous or lymphoid-specific activity, we introduced the plasmid constructs into fibroblasts and T-cell lymphomas. Although activity was easily detected in plasmacytoma, no activity was detected in either HeLa cells or in the mouse T-cell lymphomas BW5147 and EL4 (Fig. 2b). Therefore, like the IgH-intron enhancer<sup>4</sup>, the 3' enhancer is active in plasmacytoma, but not active in T cells or nonlymphoid cells in these transient transfection assays.

The sequence of the *StuI-EcoRV* fragment which encompasses enhancer activity was determined and is shown in Fig. 3. It includes a stretch of GA repeats preceded by oligo(G-T),

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StuI
AGGCCTGTGTCCATGTGGCCACAGCTAGCTCCACCGCTGCCAAGCCTGTCTGTGCAGGGA
20 40 60

TTCCGGAGTGGGGCTGGGCTCAGGGCAGCCAGSATTITTTCCAGTGGAGCAGCCAGTAGCT
80 100 120

GGGCTGAGGGTCACTGCCCAACCATCCCGTGATTCTGGGTAGACCCAGGACCTGGAGCAC
140 160 180

ACACAGCCTTCTGTCTCTCCCACTTCCCTGGGGGTGTITGGACCTTGCTATCTCTGTTC
200 220 240

ATTCCCTCTCAGGGTCAGATTTCAGCAGTGTGCGATAAATGGGAAACACTGAATTCATGG
EcoRI
260 280 300

TCACCGTTAGGGCTCATGGAAACCCCCAGCGCCCATCCCCAAGCTGTCTCAACTGGCCAGG
320 340 360

PstI PstI
GTGGGGTGAACCTGCACCTCGAGGTCGGGGAATCATTGATGCTATTTTGGCTCAGAGAA
380 420

TGAGAAACATGTTTTCTGCATGGAACAAACATCCATTTCATTTGCCCCGAGGGCCAAC
440 460 480

CCTGCACAGAGTGAAGTCTCGGCAGGTGCTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTG
500 520 540

GAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA
560 580 600

GACAGACAGACAGAGAGAGAGAGACAGAGAGAGACACACACAGAGACAGAGAGACAGAGAGA
620 640 660

GACAGAGACAGAGAGAGAGACACACACAGAGAGACAGAGAGAGAGAGAGAGATTTGTGA
680 700 720

EcoRV
GCAAGCCCTCTTTTCAGGTACTGATATC
740

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HOMOLOGIES			
Octanucleotide			$\mu$ E5
IgH-3'	456-ATTTCGCA <sup>2</sup>	IgH-3' (invert)	386-CACCTGCAGC
Consensus	ATTTCGCA <sup>2</sup>	IgH-intron	CACCTGCAGC
$\mu$ E1			AP-1
IgH-3'	346-GTCAACCTGGCC	IgH-3'	492-TGAGTCA
IgH-intron	GTCAAGATGGCC	Consensus	TGAGTCA
AP-2			AP-4
IgH-3' (invert)	214-CCCCAGGG	IgH-3' (invert)	345-CAGCTGTGG
Consensus	CCCCAGGC	Consensus	CAGCTGTGG
IgH-3'	323-CCCCAGCC		

FIG. 3 Sequence of the rat IgH 3' enhancer (IgH-3'). The sequence of both strands was determined by the dideoxy-chain-termination method. Homologies or identities to regions of the mouse IgH-intron enhancer (IgH-intron) are boxed; those to AP-1, AP-2 or AP-4-binding sites<sup>25</sup> are underlined.

Comparison of the IgH 3' enhancer with the intron of the gene for IgH and simian virus 40 (SV40) enhancers revealed homologies that span several notable motifs. In particular, a perfect copy of the octanucleotide (ATTTGCAT) is present at position 456. The octanucleotide element is found in the IgH-intron enhancer, is an essential component of V<sub>H</sub> promoters<sup>4</sup> and is sufficient in appropriate assays to confer lymphoid-specific gene expression<sup>15-17</sup>. There is also a good match to another region of the mouse intron-enhancer that is implicated in lymphoid-specific transcriptional activation, that is the region surrounding the  $\mu$ E2 and  $\mu$ E5 motifs<sup>18,19</sup>, as well as significant homology to a region surrounding the NF- $\mu$ E1 binding site. As regards SV40 comparisons, there is a match to the consensus binding site for transcription factor AP-1, and homologies to the AP-2- and AP-4-binding sites. Analysis of cosmid subfragments A, B and C (Fig. 1c) indicates that the most important motifs are likely to lie across, or 3' of, the PstI site at position 378; this would include the octanucleotide,  $\mu$ E5 and AP-1 motifs. But functional assays are clearly necessary to evaluate the significance of the homologies.

The rat IgH-intron enhancer shows B cell-specific enhancer activity and >90% sequence homology to its mouse counterpart (ref. 13, and our unpublished observations). Therefore, the rat IgH locus, like the mouse  $\kappa$  light-chain locus<sup>12</sup>, contains both a J-C intron-enhancer and a 3' enhancer. We do not believe that the presence of several enhancers is peculiar to the rat IgH locus; preliminary studies indicate that a 3' IgH enhancer is also present in the mouse IgH locus. The presence of several enhancers in the immunoglobulin gene loci could simply reflect redundancy. From transgenic mouse studies<sup>20</sup>, however, it seems that the combination of VH promoter with IgH intron-enhancer is not sufficient to give properly regulated IgH expression *in vivo*. In fact, in the context of the accessibility model of immunoglobulin gene rearrangement<sup>21</sup>, it is possible that the intron-enhancer is primarily concerned with the regulation of VH-D-JH joining, and that the 3' enhancer is implicated in transcriptional regulation at later stages of differentiation.

The existence of an enhancer at the 3' end of the IgH locus could explain the transcriptional activation of the translocated *c-myc* genes characteristic of several lymphoid malignancies. In

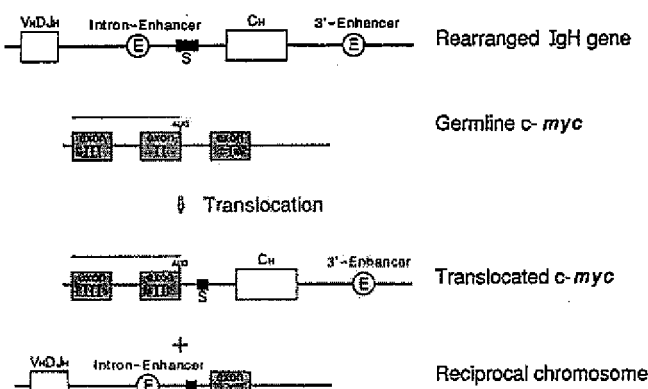


FIG. 4 The structure of a typical *c-myc*/IgH locus translocation. The precise structure of the translocation depends on the tumour, with the favoured incoming CH gene being *C<sub>ε</sub>* in rat immunocytoma, *C<sub>α</sub>* or *C<sub>γ</sub>* in mouse plasmacytoma, and *C<sub>μ</sub>* or *C<sub>γ</sub>* in Burkitt lymphoma. Similarly, the *c-myc* breakpoint can either be upstream of *c-myc* or it can be within *c-myc*, separating the noncoding exon (exon 1) from the coding region (the AUG initiator codon is in exon 2). In most cases, the breakpoint in the IgH locus is within a switch (S) region. The translocation therefore separates the IgH-intron enhancer from the coding region of *c-myc*, because they end up on reciprocal products of the translocation. But the translocated *c-myc* will become linked to an IgH 3' enhancer, which could therefore play a part in *c-myc* transcriptional activation.

many human Burkitt lymphomas, mouse plasmacytomas and rat immunocytomas, *c-myc* is translocated into the IgH locus<sup>22,23</sup>. But the location of the breakpoints on the IgH locus-bearing chromosome indicates that the IgH-intron-enhancer is unlikely to be involved in the activation of the translocated *c-myc* allele, because the protein-coding region of *c-myc* and the IgH enhancer end up on reciprocal products of the translocation<sup>24</sup>. As shown in Fig. 4, however, the translocated *c-myc* allele would typically be linked to the IgH 3' enhancer, and this enhancer could therefore be implicated in the origin or maintenance of B-cell neoplasias. □

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## A single origin of phenylketonuria in Yemenite Jews

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**PHENYLKETONURIA (PKU)** is a metabolic disease caused by recessive mutations of the gene encoding the hepatic enzyme phenylalanine hydroxylase (PAH). The incidence of PKU varies widely across different geographic areas, and is highest (about 1 in 5,000 live births) in Ireland and western Scotland, and among Yemenite Jews. A limited number of point mutations account for most of the PKU cases in the European population. Here we report that a single molecular defect—a deletion spanning the third exon of the PAH gene—is responsible for all the PKU cases among the Yemenite Jews. Examination of a random sample of Yemenite Jews using a molecular probe that detects the carriers of this deletion indicated a high frequency of the defective gene in this community. Although the deleted PAH gene was traced to 25

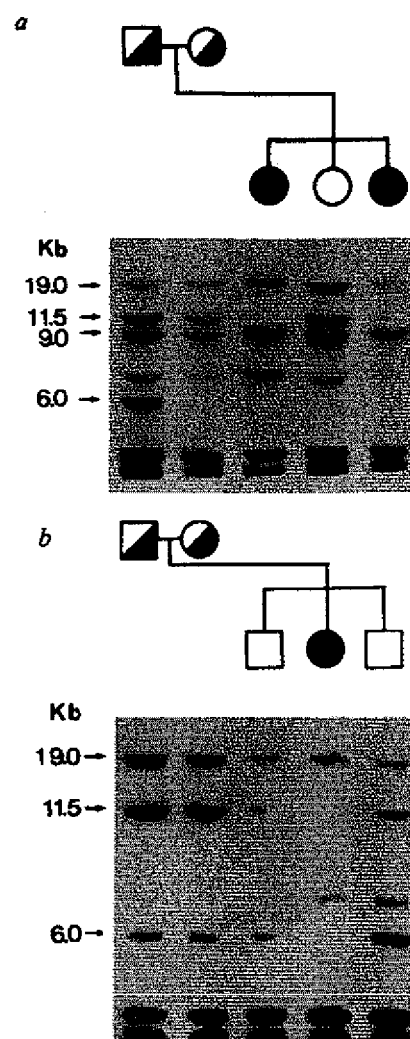


FIG. 1 Segregation of the *PvuII*(a) and *PvuII*(b) RFLPs in a non-Yemenite Jewish family (a) and a Jewish Yemenite family (b) with PKU. c. The location of the two RFLPs relative to exons 1-4 of the *PAH* gene. Normal segregation of the *PvuII*(a) alleles (6.0 and 19.0 kb) is observed in both families, whereas none of the *PvuII*(b) alleles (9.0 and 11.5 kb) are present in the DNA of the Yemenite patient. Southern blotting, hybridization and autoradiography were performed as previously described<sup>20</sup>. The cDNA probe pHPAH247 (ref. 5) was labelled using the random priming method<sup>21</sup>. Asterisks denote polymorphic restriction sites. Symbols: square, male; circle, female; filled symbol, PKU patient; half-filled symbol, PKU carrier.

different locations throughout Yemen, family histories and official documents of the Yemenite Jewish community showed that the common ancestor of all the carriers of this genetic defect lived in San'a, the capital of Yemen, before the eighteenth century.

Without PAH activity in the liver, the hydroxylation of the amino acid phenylalanine to tyrosine cannot occur, and phenylalanine accumulates in the blood and is degraded at an excessive

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# Enhancer Complexes Located Downstream of Both Human Immunoglobulin $\text{C}\alpha$ Genes

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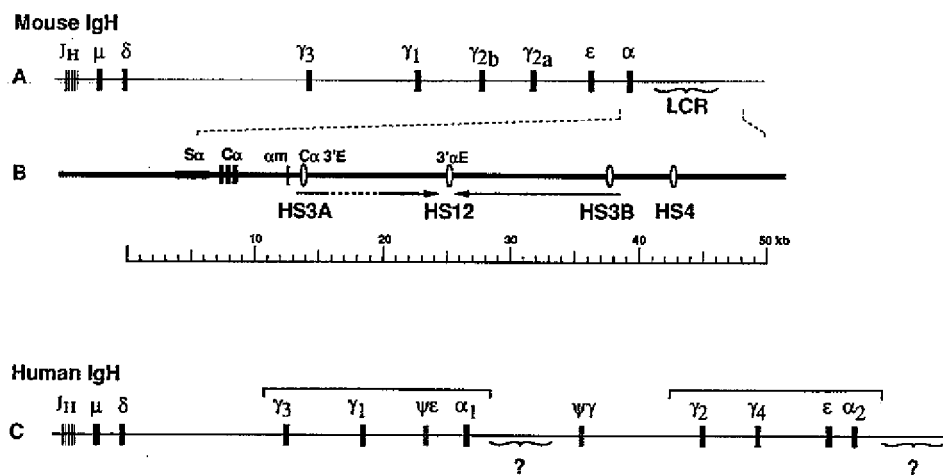
## Summary

To investigate regulation of human immunoglobulin heavy chain expression, we have cloned DNA downstream from the two human  $\text{C}\alpha$  genes, corresponding to the position in the mouse IgH cluster of a locus control region (LCR) that includes an enhancer which regulates isotype switching. Within 25 kb downstream of both the human immunoglobulin  $\text{C}\alpha 1$  and  $\text{C}\alpha 2$  genes we identified several segments of DNA which display B lymphoid-specific DNase I hypersensitivity as well as enhancer activity in transient transfections. The corresponding sequences downstream from each of the two human  $\text{C}\alpha$  genes are nearly identical to each other. These enhancers are also homologous to three regions which lie in similar positions downstream from the murine  $\text{C}\alpha$  gene and form the murine LCR. The strongest enhancers in both mouse and human have been designated HS12. Within a 135-bp core homology region, the human HS12 enhancers are ~90% identical to the murine homolog and include several motifs previously demonstrated to be important for function of the murine enhancer; additional segments of high sequence conservation suggest the possibility of previously unrecognized functional motifs. On the other hand, certain functional elements in the murine enhancer, including a B cell-specific activator protein site, do not appear to be conserved in human HS12. The human homologs of the murine enhancers designated HS3 and HS4 show lower overall sequence conservation, but for at least two of the functional motifs in the murine HS4 (a  $\kappa\text{B}$  site and an octamer motif) the human HS4 homologs are exactly conserved. An additional hypersensitivity site between human HS3 and HS12 in each human locus displays no enhancer activity on its own, but includes a region of high sequence conservation with mouse, suggesting the possibility of another novel functional element.

The regulation of human immunoglobulin heavy chain gene expression is incompletely understood, despite clinically significant conditions in which specific isotypes are inappropriately up- or downregulated, e.g., allergies due to inappropriate IgE response, and various forms of immunodeficiency associated with low IgA expression. Clearly, cytokines and interactions between B and T cells play a role in regulating isotype switching, and *cis* elements in the IgH gene locus which mediate these effects have been documented in the murine and human promoters of the sterile transcripts associated with each heavy chain constant region gene (1, 2). However, in the mouse an additional control region which contributes to regulation of isotype switching has been reported to lie downstream from  $\text{C}\alpha$ , and the corresponding region of the human heavy chain locus has not yet been investigated.

The existence of a regulatory region downstream from murine  $\text{C}\alpha$  was originally inferred when it was found that plasmacytomas which had undergone spontaneous deletions of the only heavy chain enhancer then known, which lies in the intron between JH and  $\text{C}\mu$ , nevertheless remained capable of high level immunoglobulin secretion (3–6). Conversely, a myeloma subclone which retained the intronic enhancer but lost a segment of DNA downstream from the murine  $\text{C}\alpha$  gene was found to have markedly reduced its heavy chain gene expression (7). A systematic search in the homologous region of the rat heavy chain locus revealed an enhancer (8), and a homologous mouse enhancer designated 3'  $\alpha\text{E}$  was found soon after (9, 10) positioned ~16 kb downstream from  $\text{C}\alpha$ . The mouse and rat 3'  $\alpha\text{E}$  segments lie in opposite orientations and are flanked by inverted repeats (9). In addition to the 3'  $\alpha\text{E}$ , Matthias and Baltimore also reported a weak enhancer in mouse lying only 4 kb downstream from  $\text{C}\alpha$  (Fig. 1 and reference 11).

F.C. Mills and N. Harindranath both made substantial contributions to this work.



**Figure 1.** Comparison of IgH loci of mouse and human. Line A shows a map of the murine IgH locus, from which the region downstream from C $\alpha$  is expanded in line B. The murine enhancers designated C $\alpha$ 3'E (11) and 3' $\alpha$ E (9) are shown as vertical ovals, along with the DNase I hypersensitivity site designations (12). We have distinguished the two copies of HS3 sequence as HS3A and HS3B; these are included in a large palindrome (arrows) that flanks HS12, according to the sequence analysis of Chauveau and Cogné (13). Line C shows the human IgH locus, illustrating the  $\gamma$ - $\gamma$ - $\epsilon$ - $\alpha$  duplication units (brackets) and the possibility of two regions homologous to the murine LCR.

More recently, Madisen and Groudine (12) analyzed B cell-specific DNase I hypersensitivity downstream from C $\alpha$  and identified four hypersensitive sites. HS1 and HS2 fall in the previously described 3' $\alpha$ E, whereas HS3 and HS4 lie further downstream and identify two new regions with somewhat weaker enhancer activity in transient transfection assays. The HS3 sequence is almost identical to that of the enhancer described by Matthias and Baltimore but has an inverted orientation. This reflects the fact that the sequence surrounding the HS12-3' $\alpha$ E is present in the mouse in a long inverted repeat which includes HS3 sequences at both ends (Fig. 1 and reference 13). When constructs containing HS3, HS12, and HS4 linked to a reporter gene were transfected into a B cell line, subsequently isolated stable transfectants were found to express the reporter gene in a position-independent manner. This suggested that the three enhancer sequences (HS12, HS3, and HS4) acted together as a locus control region (LCR)<sup>1</sup>. LCRs, first defined in the globin locus (14), activate large domains of chromatin in vivo (100 kb in the human  $\beta$  globin locus), and, as components of DNA constructs in transgenic mice, support gene expression proportional to the number of integrated copies. In contrast, integrated gene constructs lacking LCR sequences are variably expressed, depending on the positions of integration. LCRs typically contain several DNase I hypersensitive sites, which often represent DNA with enhancer activity. In addition to the LCRs found in the  $\beta$  globin and mouse IgH loci, LCRs have also been described as associated with macrophage-specific lysozyme, CD2, and  $\alpha/\beta$  TCR loci (15).

Analyses of the regulatory regions downstream from murine C $\alpha$  have identified several motifs which bind specific transcription factors to mediate different aspects of regulation of enhancer function. The 3' $\alpha$ E has been found to ac-

tivate transcription strongly in plasmacytomas, but only weakly in earlier B lymphoid cells. Part of this developmental change is attributable to a motif known as E5, which matches the "E-box" consensus binding site (CANNTG) characteristic for members of the basic helix-loop-helix family of transcription factors. The contribution of the E5 site to enhancer activity is inhibited in early stages of development by the dominant negative nuclear regulator Id3, which is expressed in early B lineage cells but downregulated in plasma cells (16). At least four other motifs in the 3' $\alpha$ E have been reported to contribute to enhancer activity specifically in plasmacytomas, motifs whose contribution in B cells is inhibited by BSAP (the B cell-specific activator protein), which disappears as B cells mature to plasma cells. These sites include  $\alpha$ P (17), the octamer motif (ATG-CAAAT; reference 18), a  $\kappa$ B-like site (16), and a G-rich sequence (19). In B cells, BSAP prevents the binding of the transcriptional activator NF- $\alpha$ P to the  $\alpha$ P site, and causes the octamer, G-rich, and  $\kappa$ B-like motifs to exert an active repressive influence on transcription (17, 19-21).

Apart from the motifs mediating upregulation of the 3' $\alpha$ E during maturation to plasma cells, a response element in the enhancer for activation induced by B cell receptor cross-linking has been traced to partially overlapping sites for the ETS family member Elf-1 and for members of the AP-1 transcription factor family (22). Two other motifs in the enhancer have been proposed to contribute to its regulation, but are less well documented: the  $\mu$ E1 and the  $\mu$ B motifs, which were first noted in the rat 3' $\alpha$ E and which are partially conserved in mouse. The HS3 and HS4 enhancer regions of mouse have been studied in less detail, but the HS4 enhancer apparently contains functional Oct-1 and BSAP binding sites (23).

A role for the 3' $\alpha$ E in isotype switching was revealed by experiments in which this region was replaced by a neomycin resistance gene through homologous recombination in embryonic stem cells which were then used to reconstitute the B cell population in RAG-2 knockout mice. The re-

<sup>1</sup>Abbreviations used in this paper: BAC, bacterial artificial chromosome; BSAP, B cell-specific activator protein; HSE, heat shock element; HSTF, heat shock transcription factors; LCR, locus control region.

sulting B cells showed normal V(D)J recombination but marked deficiencies in switching to IgG2a, IgG2b, IgG3, and IgE in vitro, whereas expression of IgM and IgG1 was normal (24). This observation suggests that the enhancer exerts isotype-specific effects on switch recombination, possibly through its regulation of germline transcription of the different isotypes before switch recombination.

Because the enhancer regions lying downstream from the mouse C $\alpha$  gene have been found to be important for heavy chain gene expression and isotype switching, there has been considerable interest in determining how homologous regions might regulate immunoglobulin gene expression in humans. The human heavy chain locus includes two  $\gamma$ - $\gamma$ - $\epsilon$ - $\alpha$  segments (25, 26), apparently the product of a large duplication in the primate lineage (27). Isotypes from the upstream duplication, comprising the  $\gamma$ 3- $\gamma$ 1- $\psi$  $\epsilon$ - $\alpha$ 1 constant region genes, are generally expressed at a much higher level than those of the downstream  $\gamma$ 2- $\gamma$ 4- $\epsilon$ - $\alpha$ 2 duplication. The existence of two C $\alpha$  genes in humans suggests the possibility that two 3' enhancer complexes may regulate the locus, one downstream from each C $\alpha$  gene; differences in these complexes could contribute to the differential regulation of the two duplications. Moreover, individuals who have a  $\psi$  $\epsilon$ - $\alpha$ 1- $\psi$  $\gamma$  deletion on one chromosome show reduced expression of the downstream  $\gamma$ 2 and  $\gamma$ 4 genes on that chromosome, indicating that this deletion may have removed a region which exerts distal control over at least some of the human heavy chain genes (28). Finally, the possibility that there are two 3' enhancer complexes makes the human IgH locus an attractive candidate for study because there could be interactions between two adjacent LCRs, a situation which has not been described in any other system.

Several laboratories have attempted to characterize sequences lying downstream from the two human C $\alpha$  genes, but technical difficulties have impeded this work. Gene walking downstream from the C $\alpha$  genes has been difficult, apparently because of a segment of 20-bp tandem repeats which lies almost immediately downstream of the most 3' exon of both C $\alpha$  genes. These tandem repeats, described independently by three laboratories (29–31), include the sequence GATC recognized by the isoschizomer restriction enzymes Sau3A and MboI. Since commercial human DNA libraries in  $\lambda$  phage have been constructed using genomic DNA fragments generated by partial MboI/Sau3A digestion, the repeated Sau3A sites downstream of the C $\alpha$  genes make it unlikely that library clones isolated by hybridization to C $\alpha$  probes will contain DNA downstream from the repeats.

As an initial step toward defining the role of global control regions in the activation of the human IgH genes, our laboratory has sought to determine: (a) are enhancer complexes located downstream from the human C $\alpha$  genes; and (b) how do any such human enhancers correspond to the regulatory sequences described downstream from the murine C $\alpha$  gene? Towards this end we have successfully applied several strategies other than gene walking from C $\alpha$  to obtain DNA clones extending downstream from the human

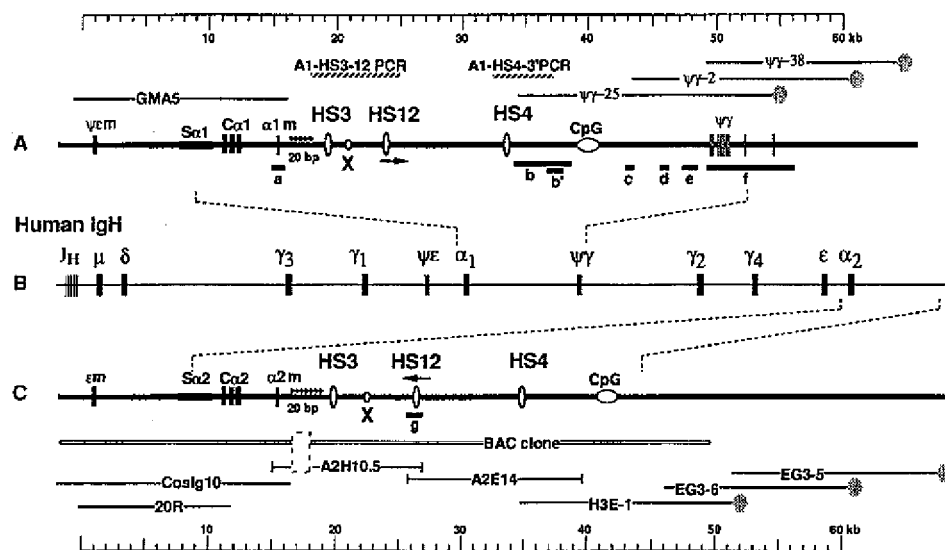
C $\alpha$  genes, enabling us, by functional analysis and sequencing, to characterize enhancer regions 3' of each C $\alpha$  gene homologous to those of the mouse HS12, HS3, and HS4.

## Materials and Methods

**Cloning of 3'  $\alpha$  Regions from Human Genomic DNA.** To obtain DNA between C $\alpha$ 1 and the previously reported  $\psi$  $\gamma$  pseudogene which lacks associated Sy sequences, we initially sought clones containing the pseudogene, which should hybridize to a C $\gamma$  probe but not to an Sy probe. We screened a commercial library of partial MboI-digested human placental DNA in the  $\lambda$ FixII phage vector (Stratagene, La Jolla, CA) with a C $\gamma$  probe: probe f (Fig. 2), a 7-kb HindIII fragment, was isolated from a pBR322 plasmid clone originally derived from  $\lambda$  phage CH-Ig-H-g-11(32) (Health Science Research Resources Bank, Osaka, Japan; e-mail: hsrhb@nih.go.jp). C $\gamma$ + plaques were replated and duplicate plaque-lift filters were hybridized with the C $\gamma$  probe and an Sy probe (1,100-bp KpnI to PstI fragment containing human Sy2; reference 33). Southern blots of BamHI-digested DNA from eight candidate clones (C $\gamma$ +, Sy-) were hybridized with a <sup>32</sup>P-labeled oligonucleotide Pseudogam-1 (see Table 1 for sequences of oligonucleotides) specific for the  $\psi$  $\gamma$  hinge region at 48°C in hybridization buffer (1 M NaCl, 0.1 M sodium phosphate, pH 7.0, 10% dextran sulfate, 10 mM EDTA, 1% SDS), followed by washing at 48°C in 1 $\times$  SSC, 10 mM EDTA, 0.1% SDS. This analysis identified three overlapping  $\psi$  $\gamma$ + clones ( $\psi$  $\gamma$ -25,  $\psi$  $\gamma$ -2, and  $\psi$  $\gamma$ -38) extending over an ~30-kb region containing the  $\psi$  $\gamma$  gene (Fig. 2).

To obtain clones for the expected homologous region downstream of the C $\alpha$ 2 region, two probes derived from the farthest upstream  $\psi$  $\gamma$  clone ( $\psi$  $\gamma$ -25) were used to rescreen two duplicate plaque lifts of the  $\lambda$ FixII library. Probe b (Fig. 2) was a 4.5-kb fragment extending from the 5' end of  $\psi$  $\gamma$ -25 to the first internal EcoRI site. Probe e was a 1.3-kb PCR fragment which lies ~2.5 kb upstream from  $\psi$  $\gamma$  and which was obtained by amplification from a  $\psi$  $\gamma$ -25 subclone containing a 10.5-kb EcoRI-XbaI fragment which extends from the farthest downstream EcoRI site in  $\psi$  $\gamma$ -25 to an artificial XbaI site at the 3' end of the phage insert. Primers used for this amplification were a reverse sequencing primer (Table 1) and elkb2, a primer based on sequence data from a  $\psi$  $\gamma$ -25 subclone. This probe contains a sequence homologous to a gene encoding the transcription factor *elk-1*, which is known to lie on the X chromosome (34). Clones EG3-6 and EG3-5 were selected for the following properties: they hybridized to probe e but not to a C $\gamma$  probe (as expected for DNA downstream of C $\alpha$ 2), and they had restriction maps distinct from that of the  $\alpha$ 1 locus and that of the *elk-1* gene on the X chromosome, as established by genomic Southern blots of DNA from a mouse/human somatic hybrid cell line (NIGMS repository No. GM06318B; Coriell Institute for Medical Research, Camden, NJ) carrying the human X chromosome as its only human DNA. Clone H3E-1 was isolated on the basis of hybridization with probe b but not with a C $\gamma$  probe. By restriction maps and Southern blots, clones H3E-1, EG3-6, and EG3-5 were found to overlap with each other, forming a contig of ~30 kb.

From genomic Southern blots, the phage contigs downstream of the two C $\alpha$  genes were found to lie ~20 kb away from clones containing the respective C $\alpha$  genes. To bridge these gaps, a bacterial artificial chromosome (BAC) library of human genomic DNA (Genome Systems, Inc., St. Louis, MO) was screened using both a C $\alpha$  membrane exon probe (Fig. 2, probe a) made by amplification from the C $\alpha$ 1 plasmid GMA5 using primer 5'TM-A1



position of a DNase I site which shows human/mouse sequence conservation. The position of a CpG island previously identified by Southern blotting is also shown (oval). The arrow under HS12 in line A indicates the orientation of this sequence, which is the same as that of the homologous mouse HS site, but opposite from the orientation of HS12 in the  $\alpha 2$  locus (line C). The thick black lines under the maps of lines A and C (single lower case letters) represent hybridization probes used in this study.

in combination with primer 3'TM-B (35) and a probe extending from the most 5' HindIII site in  $\psi\gamma$ -25 to a position  $\sim 1,300$  bp upstream (Fig. 2, probe b'). A single BAC clone was obtained, spanning  $\sim 120$  kb, from  $\sim 20$  kb upstream of  $C\gamma 2$  to 35 kb downstream from the  $C\alpha 2$  membrane exon. From this clone, designated BAC 11771, two subclones were prepared: a 10.5-kb HindIII fragment (A2H10.5) and an overlapping 14-kb EcoRI fragment (A2E14). Fragments of these subclones were found to have sequence homology to murine HS12, HS3, and HS4, and enhancer activity in a transient transfection assay (see Results).

Because several library screens were unsuccessful in identifying phage or BAC clones covering the gap between  $C\alpha 1$  and our downstream phage contig, selected DNA segments in this region were amplified by PCR. Southern blots of human genomic DNA indicated that the HS12 regions near  $\alpha 1$  and  $\alpha 2$  lie in opposite orientation. This inversion made it possible to selectively amplify  $\alpha 1$ -derived sequence from genomic DNA using two primers which both corresponded to the sense strand of the  $\alpha 2$  locus. Amplification with the upstream primer SA2.5-A2 and the downstream primer SA2.1-A2 was performed using Taq polymerase XL (PE Applied Biosystems, Foster City, CA) for 32 cycles ( $94^\circ\text{C}$  for 1 min,  $61^\circ\text{C}$  for 2 min, and  $72^\circ\text{C}$  for 10 min), and yielded the 5.5-kb A1-HS3-12 PCR fragment (Fig. 2). From this fragment a 982-bp  $\alpha 1$  HS-3 product was amplified using primers SA2.5A and SA2.6A; and 964 bp  $\alpha 1$ HS12T and 892 bp  $\alpha 1$ HS12B fragments were amplified using primers SA2.1A and SA2.2B. For comparisons between enhancer activities of corresponding  $\alpha 1$  and  $\alpha 2$  fragments, the homologous 1070-bp A2HS12 PCR product was generated with the same primer pair but using plasmid pEH1.3 as template. In experiments seeking the  $\alpha 1$  homolog of HS4, amplifications exploiting the inversion were unsuccessful. As an alternative strategy, we prepared an  $\alpha 1$ -specific genomic template for PCR: a 23-kb HindIII fragment from the human myeloma HS Sultan (American Type Culture Collection, Rockville, MD) which Southern blots indicated extended from the  $\alpha 1$  membrane exon to a HindIII site in our  $\psi\gamma$ -25 clone, including DNA homologous to HS4. The corresponding regions

from  $\alpha 2$  fall in 12-kb and 14-kb HindIII fragments. A 23-kb preparative electrophoresis fraction of HindIII-digested DNA from HS Sultan was used as template for amplification with primers SA8.A and SA11.B based on sequence from the  $\alpha 2$  locus. The resulting 4.2-kb fragment (A1-HS4-3'PCR; Fig. 2) was cloned into a  $\lambda$ ZAP Express phage vector (Stratagene), yielding the clone  $\alpha 1$ HS4-4.5; the cloned insert showed a restriction map consistent with the  $\alpha 1$  locus and distinct from the  $\alpha 2$  locus. From the 4.2 A1-HS4-3'PCR fragment a 468-bp segment containing the  $\alpha 1$  HS4 region was amplified using primers SA.8A and SA.9B; this segment was used for sequence analysis and enhancer studies. With the same primer pair, the corresponding  $\alpha 2$  fragment A2-HS4 was generated using plasmid pA2E14 as template in order to enable meaningful  $\alpha 1$  versus  $\alpha 2$  comparisons of HS4 enhancer activity.

**DNase I Hypersensitive Site Analysis.** Nuclei were prepared and digested with DNase I according to a previously described protocol (36). K562 and HS Sultan cells (both obtained from American Type Culture Collection) were grown to densities of  $5\text{--}8 \times 10^5$  cells/ml. For each experiment,  $3\text{--}6 \times 10^8$  cells were harvested, lysed by addition of NP-40, centrifuged through a 1.7 M sucrose cushion, and resuspended in 5 ml; 450- $\mu\text{l}$  aliquots of suspended nuclei were treated with serially diluted DNase I (Boehringer-Mannheim, Indianapolis, IN) to give final DNase I concentrations of 0–8  $\mu\text{g}/\text{ml}$ . Nuclei were digested with DNase I for 3 min at  $25^\circ\text{C}$ . For one experiment HS Sultan nuclei were digested with the restriction endonuclease SspI (New England Biolabs, Beverly, MA) for 15 min at  $37^\circ\text{C}$ . DNase I (or SspI) digestion was terminated by adding 50  $\mu\text{l}$  1% SDS, 100 mM EDTA. DNA samples were deproteinized for 5–48 h at  $37^\circ\text{C}$  using proteinase K (Boehringer-Mannheim) at a final concentration of 100  $\mu\text{g}/\text{ml}$ . DNA was purified by phenol/chloroform extraction and ethanol precipitation, resuspended in 50–100  $\mu\text{l}$  deionized water, and DNA concentrations were measured using a Fluorometer (TKO 100; Hoeffer, San Francisco, CA). 5- $\mu\text{g}$  DNA samples were digested for 5–24 h in 50  $\mu\text{l}$  of appropriate restriction enzyme buffer with BglII, EcoRI, or HindIII (New England Biolabs). To

**Figure 2.** Regulatory loci downstream of human  $C\alpha 1$  and  $C\alpha 2$ . Lines A and C, based on this study, show an expanded map of the region downstream of  $C\alpha 1$  and  $C\alpha 2$ , respectively, as well as available DNA clones, which are shown above ( $\alpha 1$ ) or below ( $\alpha 2$ ) the line; phage clones are marked with diagrammatic phage heads, while the subclones of PCR-amplified segments A1-HS3-12 and A1-HS4-3' are drawn with hatched lines; and a BAC clone is drawn as a double line, containing a deletion (dashed box). Vertical ovals mark DNase I sites demonstrating enhancer activity and named according to the homologous murine HS sites. A series of small triangles identifies the 20-bp repeats located downstream from human  $C\alpha$  genes. X marks the

**Table 1.** Sequences of Oligonucleotides Used in This Study

Oligonucleotide name	Sequence
Pseudogam-1	AGATGCCCACCATGTCAAGT
Reverse sequencing primer	AACAGCTATGACCATG
elkb2	TAAGCTGTCTGAGAGAAAGTTGGGGGAGG
5'TM-A1	CTGTTTACACGAGTCTGGGCCTGG
3'TM-B	TCCAAGAGGTTTCTCCACACTTCC
SA2.5-A2	<u>ggccggtacc</u> GGATCCCCGGTTCCTGATCACTG
SA2.1-A2	<u>ggccggtacc</u> CTTCCTGCCAACCTGGGGGCTG
SA2.5A	<u>ggccacgcgt</u> GGATCCCCGGTTCCTGATCACTG
SA2.6A	<u>ggccacgcgt</u> CCACAGTCACTGCCAGATGCTC
SA2.1A	<u>ggccacgcgt</u> CTTCCTGCCAACCTGGGGGCTG
SA2.2B	<u>ggccacgcgt</u> GGCTTTTGGCCAGTCTCTCTAC
SA11.B	CAGTGCCCCCAACCCAGGACGCCAGCC
SA.8A	<u>ggccacgcgt</u> CGCTCGCTGCCCCACTCAGGAGG
SA.9B	<u>ggccacgcgt</u> CTCCTAGCAGGGTCTCCTCCCTGG
$\beta$ 5PR-A	GAGGAGAAGTCTGCCGTTACTGCCC
$\beta$ 3PR-B	GGCACAATCCAGATGCTCAAGGCC

All primer sequences are presented 5' to 3'. For certain primers derived from human genomic DNA sequence, additional nucleotides were added at the 5' end to provide restriction sites; these nongenic nucleotides are written in lowercase letters, with the restriction site underlined. All oligonucleotides were prepared in the Facility for Biotechnology Research (Center for Biologics Evaluation and Research, Bethesda, MD).

assess the *Ssp*I sensitivity of the  $\beta$  globin locus in HS Sutan nuclei, a 1,511-bp human  $\beta$  globin probe was amplified from total human genomic DNA using primers  $\beta$ 5PR-A and  $\beta$ 3PR-B. Restriction-digested samples, together with  $^{32}$ P-labeled size markers, were electrophoresed, blotted, and hybridized with the probes indicated in the figure legends. After washing the membranes, radioactive images were obtained with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**Enhancer Assays.** To analyze DNA fragments for enhancer activity, we used the luciferase reporter plasmid pGL3 (Promega Corp., Madison, WI), modified so that the SV40 promoter between *Bgl*II and *Hind*III sites was replaced by a 76-bp *V $\kappa$*  promoter (37) containing an octamer motif and TATA box. This plasmid, named pGL3-*V $\kappa$* , served as an enhancerless, promoter-only control. Fragments to be assayed for enhancer activity were blunt-ended with Klenow fragments of DNA polymerase and ligated with *Mlu*I linkers, or amplified with primers incorporating an *Mlu*I (or *Kpn*I) site; the fragments were then cloned into the *Mlu*I (or *Kpn*I) site in the polylinker upstream of the promoter in pGL3-*V $\kappa$* . Plasmid DNAs for transfection were twice purified on a CsCl gradient.

Approximately  $5 \times 10^6$  cells in mid-log phase were washed with PBS and electroporated with either 5  $\mu$ g of promoter-only control construct or equimolar amounts of enhancer test constructs along with 5  $\mu$ g of CMV- $\beta$ -galactosidase plasmid (pCMV $\beta$ ; Clontech, Palo Alto, CA) as an internal control. Each plasmid was electroporated in triplicate in 400  $\mu$ l PBS using an Electro Cell Manipulator (model 600; BTX Inc., San Diego, CA) at 200 V, 900  $\mu$ F capacitance, and 13  $\Omega$  resistance in a 0.2-cm electroporation cuvette. Immediately after transfection cells were returned to culture medium. 24 h after transfection, the cells were harvested, washed with PBS, and lysed in 50  $\mu$ l reporter lysis buffer (Promega Corp.) at room temperature for 20 min. After centrifugation at

15,000 rpm for 5 min, 15  $\mu$ l of supernatant extracts were assayed for  $\beta$ -galactosidase using Galacto-Light Plus Chemiluminescent Reporter Assay kit (Tropix Inc., Bedford, MA) according to the manufacturer's instructions. To assay for luciferase activity, 35  $\mu$ l of cell extracts was incubated with 100  $\mu$ l of luciferase substrate [470  $\mu$ M luciferin in 20 mM tricine, 1.07 mM ( $\text{MgCO}_3$ )  $\times$   $\text{Mg}(\text{OH})_2 \times \text{H}_2\text{O}$ , 2.67 mM  $\text{MgSO}_4$ , 0.1 mM EDTA, 33.33 mM DTT, 270  $\mu$ M coenzyme A, and 540  $\mu$ M ATP]. For both  $\beta$ -galactosidase and luciferase assays light output was detected in a luminometer (Dynatech Instruments, Chantilly, VA).

**Sequence Analysis.** A PCR-based methodology employing  $^{33}$ P-labeled ddNTPs was used for sequencing reactions (ThermoSequenase kit; Amersham Life Science, Arlington Heights, IL). All samples were amplified for 50 cycles (95°C for 30 s; 60°C for 30 s; 72°C for 60 s). Sequencing reactions were electrophoresed on 6% gels, dried, autoradiographed, and read manually. All reported sequences were read on both A and B strands, except for the sequences of the HS12 59-bp repeats, which, despite several attempts employing various strategies, could be read only on one strand.

## Results

**Cloning DNA Downstream of *Ca1* and *Ca2*.** Because attempts to clone the human 3' *C $\alpha$*  regions using strategies based on gene walking from *C $\alpha$*  or cross-species hybridization had failed in other laboratories, we used an alternative strategy based on the fact that a *C $\gamma$* -like pseudogene ( *$\psi\gamma$* ) has been described downstream of human *Ca1* (32, 38). We reasoned that we could clone DNA downstream of *Ca1* by walking upstream from  *$\psi\gamma$* . The similarity in the restriction maps reported downstream of the two *C $\alpha$*  genes

based on Southern blotting (26) furthermore suggested that these regions would be highly similar to each other. Therefore, probes derived by walking upstream from  $\psi\gamma$  should also hybridize to clones containing DNA from downstream of  $C\alpha 2$ .

By screening a phage library for clones that hybridized to  $C\gamma$  but not  $S\gamma$ , and then screening those clones with a  $\psi\gamma$ -specific oligonucleotide (see Materials and Methods), we obtained three overlapping clones spanning  $\sim 30$  kb (Fig. 2). To obtain clones covering the corresponding region from the  $\alpha 2$  locus, the library was then rescreened with probes b and e (Fig. 2). Clones deriving from downstream of  $C\alpha 2$  were selected on the basis of restriction site differences between the  $\alpha 1$  and  $\alpha 2$  which had been established by analysis of genomic Southern blots and comparisons with our cloned DNA from the  $\alpha 1$  duplication. Three clones deriving from the  $\alpha 2$  locus and spanning  $\sim 30$  kb were obtained (Fig. 2).

To estimate the distance between our two phage contigs and the corresponding  $C\alpha$  genes, we performed genomic Southern blot experiments using a panel of restriction enzymes with known sites in the  $C\alpha$  loci and in our contigs. For example, co-migrating  $Bgl$ III bands of  $\sim 35$  kb were found to hybridize to both probe a (from the  $C\alpha$  membrane exon  $\alpha m$ ) and probe b (Fig. 2); these experiments suggested a gap of  $\sim 20$  kb between each contig and the corresponding membrane exon of  $C\alpha$  (data not shown). For the  $\alpha 2$  locus, the gap was bridged by a BAC clone (see Materials and Methods). The corresponding regions from the  $\alpha 1$  locus have resisted direct cloning and have been obtained by PCR using primers designed from the sequence of the  $\alpha 2$  locus, as described in Materials and Methods.

**DNase I Hypersensitive Site Analysis.** To map potential regulatory sequences downstream of the  $C\alpha$  genes, we used fragments from the cloned DNA downstream of the  $C\alpha 2$  gene to search for DNase I hypersensitivity sites; in cells where such enhancers or promoters are active, they generally are hypersensitive to endonucleases, apparently because binding of transcription factors disrupts the nuclease protection afforded by nucleosomes. Intact nuclei from the human myeloma HS Sultan were incubated briefly with various concentrations of DNase I; DNA samples purified from these treated nuclei were then analyzed using several Southern blot strategies in order to localize the positions of DNase I cleavage. The analyses were complicated by the fact that all of the probes we used hybridized to both the  $\alpha 1$  and  $\alpha 2$  loci, but various restriction map differences between the two loci allowed us to position all the hypersensitivity sites with respect to restriction sites mapped from our clones.

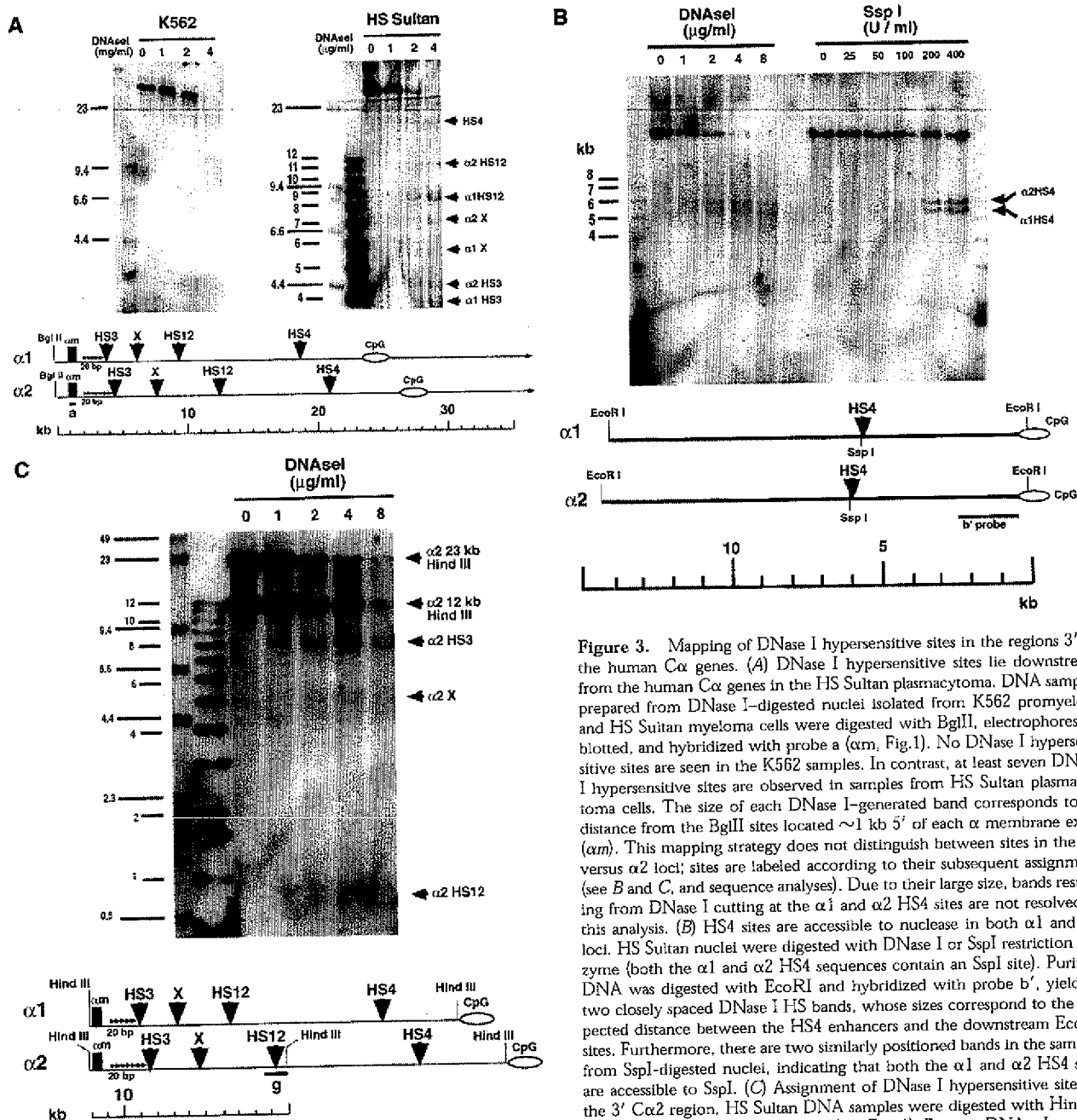
Fig. 3 A demonstrates analyses of DNA from the promyelocyte K562 line, representative of a cell not expressing immunoglobulin genes, and the myeloma HS Sultan line. The DNase I-treated DNA was digested with  $Bgl$ III, which cuts  $\sim 1$  kb upstream of the membrane exon of both  $C\alpha$  genes; the blots were hybridized with a probe corresponding to this exon. Since the next downstream  $Bgl$ III site is  $>20$  kb away, this strategy can display hypersensitivity sites

over this wide distance from the  $\alpha$  membrane exon. The blot demonstrates at least seven hypersensitivity sites, which were subsequently assigned to the  $\alpha 1$  or  $\alpha 2$  locus by other blotting experiments and were named according to sequence similarity to the homologous murine regions as described below. The sites we have tentatively designated  $\alpha 1X$  and  $\alpha 2X$  do not correspond to any reported murine enhancer sequence. None of these sites were visible in the DNA from K562, in which the enhancer region is expected to be inactive.

The  $Bgl$ III blot of HS Sultan DNA fails to resolve the two HS4 sites because they are too far away from the  $Bgl$ III sites. The two HS4 sites were resolved by an alternative Southern blot strategy employing  $Eco$ RI digests of the DNA from DNase I-digested nuclei (Fig. 3 B). To determine which of the two resulting bands represented HS4 from the  $\alpha 1$  versus  $\alpha 2$  loci, we exploited the observation from our sequence analysis that recognition sites for the restriction enzyme  $Ssp$ I lie in each HS4 site. Since many regulatory regions accessible to DNase I are also accessible to restriction endonucleases, we digested HS Sultan nuclei with  $Ssp$ I and localized the cleavage sites by isolating the DNA, digesting with  $Eco$ RI, and hybridizing Southern blots with probe b' as shown in Fig. 3 B. From the map positions of the  $\alpha 1$  and  $\alpha 2$   $Ssp$ I sites we assigned the  $Ssp$ I hypersensitivity bands as shown in Fig. 3. A control experiment on the same DNA isolated from  $Ssp$ I-digested HS Sultan nuclei demonstrated that  $Ssp$ I recognition sites in the  $\beta$  globin locus were not cut, indicating that the  $Ssp$ I sites associated with HS4 were indeed hypersensitive in HS Sultan cells (data not shown; see Materials and Methods).

Fig. 3 C illustrates an experiment which allowed assignment of several HS sites to the  $\alpha 2$  locus rather than  $\alpha 1$ . DNA samples from DNase I-treated HS Sultan nuclei were digested with  $Hind$ III and hybridized with a probe for the HS12 site derived from the  $\alpha 2$  locus. Although this probe hybridizes to both the  $\alpha 1$  and  $\alpha 2$  loci, in this DNA the  $\alpha 1$  band is  $\sim 23$  kb, so large that any fragments from this locus that were generated by  $Hind$ III and DNase I and which hybridized to the probe would be larger than the 12-kb band representing the  $\alpha 2$  locus. Thus all HS bands  $<12$  kb derive from  $\alpha 2$ . This blot therefore defines the position of the HS12, X, and HS3 sites in the  $\alpha 2$  locus. By implication, the other HS sites in the  $Bgl$ III blot of Fig. 3 A must derive from  $\alpha 1$ . (It should be noted that a common allele in other DNA samples shows an additional polymorphic  $Hind$ III site which cuts within the 23 kb corresponding to the HS Sultan band; this allele would have confounded the above strategy, but was absent in HS Sultan.)

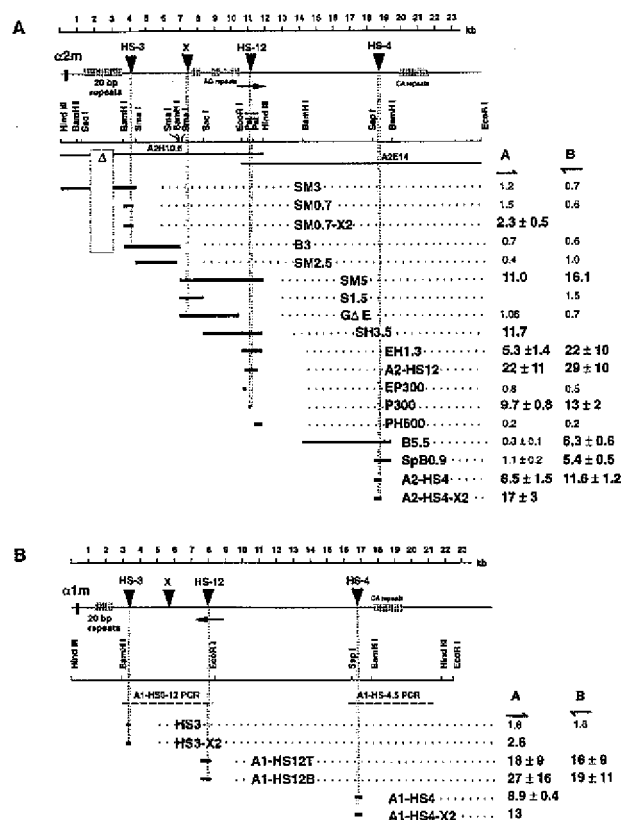
**Enhancer Function.** To analyze enhancer activity in the cloned DNA downstream of the  $C\alpha 2$  locus, fragments containing one or more DNase I hypersensitive sites were subcloned into a luciferase reporter gene driven by a  $V\kappa$  promoter as described in Materials and Methods. Luciferase activity in each sample was normalized to the  $\beta$ -galactosidase activity of the promoter-only control plasmid, and expressed as fold-increase over luciferase activity of that control plasmid.



**Figure 3.** Mapping of DNase I hypersensitive sites in the regions 3' of the human  $\alpha$  genes. (A) DNase I hypersensitive sites lie downstream from the human  $\alpha$  genes in the HS Sultan plasmacytoma. DNA samples prepared from DNase I-digested nuclei isolated from K562 promyeloid and HS Sultan myeloma cells were digested with BglII, electrophoresed, blotted, and hybridized with probe a ( $\alpha$ m, Fig. 1). No DNase I hypersensitive sites are seen in the K562 samples. In contrast, at least seven DNase I hypersensitive sites are observed in samples from HS Sultan plasmacytoma cells. The size of each DNase I-generated band corresponds to its distance from the BglII sites located  $\sim 1$  kb 5' of each  $\alpha$  membrane exon ( $\alpha$ m). This mapping strategy does not distinguish between sites in the  $\alpha 1$  versus  $\alpha 2$  loci; sites are labeled according to their subsequent assignment (see B and C, and sequence analyses). Due to their large size, bands resulting from DNase I cutting at the  $\alpha 1$  and  $\alpha 2$  HS4 sites are not resolved in this analysis. (B) HS4 sites are accessible to nuclease in both  $\alpha 1$  and  $\alpha 2$  loci. HS Sultan nuclei were digested with DNase I or SspI restriction enzyme (both the  $\alpha 1$  and  $\alpha 2$  HS4 sequences contain an SspI site). Purified DNA was digested with EcoRI and hybridized with probe b', yielding two closely spaced DNase I HS bands, whose sizes correspond to the expected distance between the HS4 enhancers and the downstream EcoRI sites. Furthermore, there are two similarly positioned bands in the samples from SspI-digested nuclei, indicating that both the  $\alpha 1$  and  $\alpha 2$  HS4 sites are accessible to SspI. (C) Assignment of DNase I hypersensitive sites to the 3'  $\alpha 2$  region. HS Sultan DNA samples were digested with HindIII and hybridized with probe g ( $\alpha 2$  HS12, Fig. 1). Because DNase I-generated bands from the  $\alpha 1$  region which hybridize to this probe are expected to be larger than the 12-kb  $\alpha 2$  HindIII fragment, all bands  $< 12$  kb must result from DNase I cutting in the 3'  $\alpha 2$  region, with the size of these bands corresponding to their distance from the 3' end of the 12-kb  $\alpha 2$  HindIII fragment. This analysis allows assignment of three DNase I sites to the  $\alpha 2$  locus, thus making it possible to assign the other hypersensitive sites seen in the BglII analysis of Fig. 2 A to the  $\alpha 1$  locus.

The luciferase assays (Fig. 4) revealed strong enhancer activity in the 5-kb SmaI-HindIII fragment (SM5) which was found to contain sequence homologous to murine HS12 (see below). Within this fragment, enhancer activity seemed to be confined to the 1.3-kb EcoRI-HindIII fragment (EH1.3) containing the HS12 site. This segment was further cut into an upstream 0.3-kb EcoRI-PstI fragment

(EP300), a 0.3-kb PstI-PstI fragment (P300), and a 0.6-kb PstI-HindIII fragment (PH600). Of these, only the P300 fragment, which contained HS12, showed enhancer activity. However, it should be noted that the enhancer activity of P300 was less than that measured for a larger PCR-generated fragment A2HS12. Thus it is possible that additional elements that do not show intrinsic enhancer activity when



**Figure 4.** Enhancer activity of selected regions downstream of human  $\alpha 1$  and  $\alpha 2$  genes. (A) Analysis of the locus downstream of  $\alpha 2$ , which was studied in detail. The map shows the position of DNase I sites; below this are diagrammed the restriction sites defining the boundaries of each fragment tested for enhancer activity by insertion into pGL3-V $\kappa$ , transfection into the human myeloma HS Sultan, and assay of resulting luciferase activity, as described in the text. The enhancer activities are given for constructs in the A orientation (the same orientation with respect to transcribed strands of immunoglobulin and luciferase) or the opposite B orientation, where examined. The luciferase activities were normalized to  $\beta$ -galactosidase activity encoded by a cotransfected plasmid, and expressed as fold-increase over the activity of an enhancerless control plasmid. For fragments showing enhancer activity, assays were performed at least in triplicate, and standard deviations are given. (B) Comparable analysis of selected fragments amplified from the homologous locus downstream from  $\alpha 1$ .

isolated in constructs can nevertheless augment the activity of the core HS12 enhancer lying in the P300 fragment. Furthermore, the EH1.3 fragment, but not the slightly shorter A2HS12 fragment, showed significantly less enhancer activity in the A orientation, suggesting the possibility of inhibitory sequences located near one of the ends of EH1.3.

Two fragments containing the  $\alpha 2$  HS3 site showed no significant enhancer activity in the luciferase construct, but a plasmid in which the HS3-containing 0.7-kb BamHI-SmaI fragment was dimerized showed consistent low activity (Fig. 4, SM0.7-X2). The homologous murine enhancer was reported to show greater activity with a c-myc promoter than with an immunoglobulin V $\lambda$  promoter (12), but we found no significant increase in enhancer activity

when the V $\kappa$  promoter was replaced by a human c-myc promoter (data not shown; construct described in Materials and Methods).

Two restriction fragments, B5.5 and SpB0.9, containing the  $\alpha 2$  HS4 site demonstrated significant stimulation of luciferase activity, but only when cloned in the B (inverted) orientation (Fig. 4 A). When these fragments were oriented so that the DNA strand continuous with the transcribed strand in the luciferase gene corresponded to the transcribed strand of the immunoglobulin gene in genomic DNA (the A orientation), luciferase activity was no greater than that from the promoter-only plasmid. Unexpectedly, a smaller fragment (the 468-bp PCR-generated fragment designated A2-HS4, designed to span the sequence showing the strongest homology to the murine HS4 site) was found to give a strong activation of luciferase activity. The stronger activation of luciferase conferred by the short A2-HS4 PCR fragment compared with that of the longer SpB0.9 fragment (in the same A orientation) suggests that the latter may contain an inhibitory sequence that is either position- or orientation-dependent. This possibility is currently being explored.

Because of the lack of genomic clones spanning the HS3, HS12, and HS4 regions of  $\alpha 1$  locus, fragments corresponding to each of these sites were obtained by PCR and analyzed; and, to facilitate  $\alpha 1$  versus  $\alpha 2$  locus comparisons, the same primer pairs were used to generate corresponding fragments from the  $\alpha 2$  locus. As shown in Fig. 4, the activity of each HS site fragment from  $\alpha 1$  was similar to that from the  $\alpha 2$  locus (compare SM0.7-X2  $\alpha 2$  versus HS3-X2  $\alpha 1$ ; A2HS12  $\alpha 2$  versus A1HS12T  $\alpha 1$ ; and A2-HS4  $\alpha 2$  versus A1-HS4  $\alpha 1$ ). The PCR amplification of the HS12 site yielded two fragments, designated A1HS12T (top) and A1HS12B (bottom), which differ in size by  $<0.1$  kb and may represent alleles (see below). Both fragments showed substantial enhancer activity (Fig. 4 B).

Since the mouse 3'  $\alpha$  enhancers show different activation patterns during B cell differentiation, with HS4 being activated at the pre-B cell stage, while HS12 and HS3 are only active in mature B cells, we examined the activity of the human 3'  $\alpha 2$  enhancers in a range of human and mouse B cell lines (Table 2). The pattern of activation for the human HS12 enhancer is similar to that of mouse HS12; i.e., human HS12 is inactive in the human pro-B cell line FLEB-14 and the mouse pre-B cell line 18-81, but functions in the human mature B cell line Raji, as well as three plasmacytomas (HS Sultan, human; S194, mouse; and MOPC 315, mouse). HS3 is also inactive in mouse 18-81 pre-B cell line, but shows modest activity in most of the more mature lines tested. HS3 shows surprisingly strong activity in the mouse S194 myeloma, indicating that unknown factors varying between cell lines at similar stages of differentiation can modulate the activity of this enhancer. Finally, the HS4 enhancer shows strong activity in the human pro-B cell line FLEB14, and is also variably active in all of the more mature cell lines (except 18-81) in which this enhancer was assayed in the B orientation.

**Sequence Analysis.** The nucleotide sequence of all DNase I hypersensitivity sites was determined, revealing  $\sim 99\%$  se-

**Table 2.** Cell Specificity of Human  $\alpha 2$  Enhancer Elements

Cell line	HS12 (EH1.3)		HS3 (SM0.7)			HS4 (SpB0.9)	
	A	B	A	B	X2	A	B
<b>Human</b>							
FLEB 14 (pro B)	1.9 $\pm$ 0.1	1.9 $\pm$ 0.1	ND	ND	ND	<b>2.8 <math>\pm</math> 0.2</b>	<b>18.4 <math>\pm</math> 2.4</b>
Raji (Mature B)	<b>3.1 <math>\pm</math> 2.2</b>	<b>3.2 <math>\pm</math> 1.8</b>	0.7 $\pm$ 0.2	1.0 $\pm$ 0.3	<b>2.8 <math>\pm</math> 0.5</b>	1.2 $\pm$ 0.1	<b>2.7 <math>\pm</math> 0.5</b>
HS Sultan (Plasmacytoma)	<b>5.3 <math>\pm</math> 1.4</b>	<b>22 <math>\pm</math> 10</b>	1.54	0.59	<b>2.3 <math>\pm</math> 0.5</b>	1.1 $\pm$ 0.2	<b>5.4 <math>\pm</math> 0.5</b>
<b>Mouse</b>							
18-81 (Pre-B)	0.9 $\pm$ 0.2	1.1 $\pm$ 0.3	1.2 $\pm$ 0.1	0.8 $\pm$ 0.2	1.6 $\pm$ 0.5	0.5 $\pm$ 0.1	0.9 $\pm$ 0.2
S 194 (Plasmacytoma)	<b>5.3 <math>\pm</math> 0.2</b>	<b>14.4 <math>\pm</math> 1.0</b>	<b>8.5 <math>\pm</math> 0.8</b>	<b>2.6 <math>\pm</math> 0.4</b>	<b>16.3 <math>\pm</math> 0.3</b>	<b>7.2 <math>\pm</math> 1.4</b>	<b>28.4 <math>\pm</math> 2.3</b>
MOPC 315 (Plasmacytoma)	<b>5.9 <math>\pm</math> 1.1</b>	<b>16.9 <math>\pm</math> 1.9</b>	1.4 $\pm$ 0.2	0.7 $\pm$ 0.2	<b>2.9 <math>\pm</math> 1.9</b>	1.3 $\pm$ 0.6	<b>5.8 <math>\pm</math> 0.9</b>

The numbers given represent the fold-increase of luciferase activity seen with a promoter-only control plasmid, with standard deviation. Numbers in bold were judged to represent significant enhancer activity.

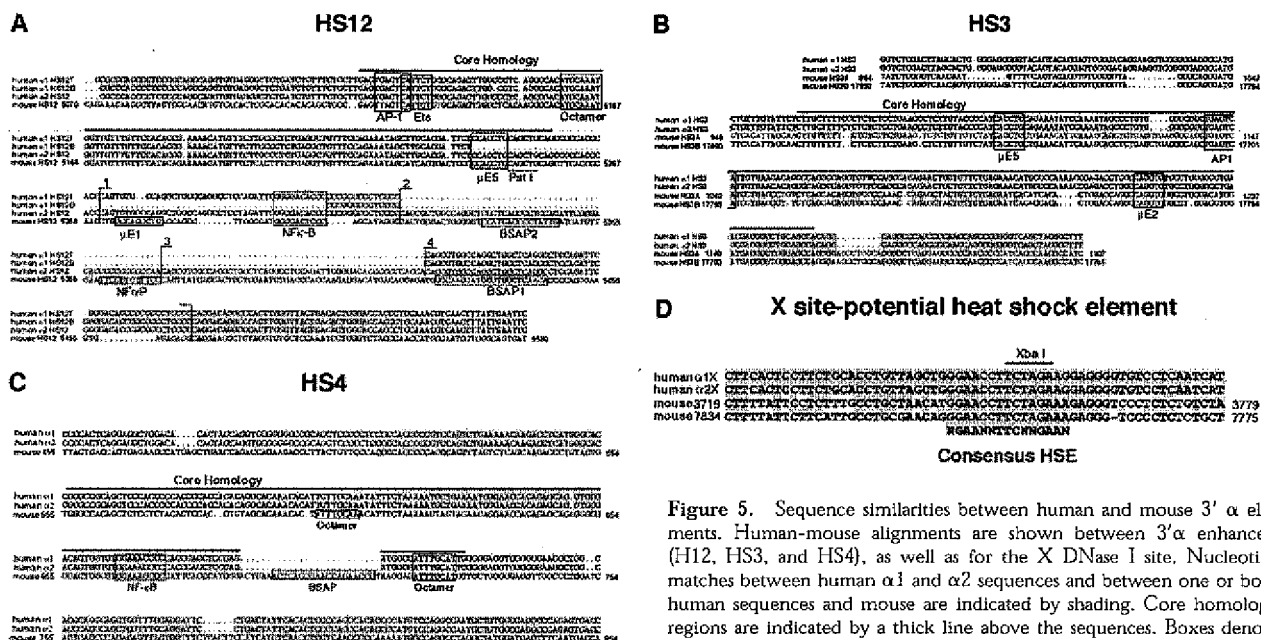
quence identity between the human  $\alpha 1$  and  $\alpha 2$  elements, and similarity between the human and mouse enhancers ranging from 74 to 90%.

HS12, the strongest enhancer, showed 90% sequence identity to the homologous murine enhancer over a 135-bp core homology (Fig. 5 A). In the  $\alpha 2$  locus, four tandem repeats with a 59-bp consensus sequence lie immediately upstream of the HS12 core. However, this sequence has been inverted in Fig. 5 A, to facilitate comparison with the homologous  $\alpha 1$  sequence in opposite orientation. In the corresponding region of  $\alpha 1$  (which, due to the inversion, lies downstream of the core homology region) a 115-bp deletion removes the second and third repeats; the  $\alpha 1$ HS12B region shows an additional deletion of 70 bp. The core homology region includes several of the functional motifs identified in the murine enhancer: the AP1-Ets site reported to confer responsiveness to B cell receptor cross-linking (22, 39); an exact octamer sequence (ATGCAAT); and a  $\mu$ E5 site (except in the  $\alpha 1$ HS12B sequence, in which the  $\mu$ E5 is missing owing to the 70-bp deletion). The sequences of these three motifs from the human HS12 are identical to their murine homologs except for a single base change in the AP1 site which causes the human sequence to exactly match the consensus AP1 site, where the murine motif has one mismatch. The murine element designated  $\mu$ E1 (39), which has never been thoroughly documented even in the murine enhancer, is poorly conserved in the human homologs. Although the murine binding site for NF- $\kappa$ B lies outside the 135-bp region of strongest sequence similarity, a reasonable match to consensus for this element is found in a position roughly homologous to the murine  $\kappa$ B site in the  $\alpha 1$ HS12T and  $\alpha 2$ HS12 sequences, but is part of the 70 bp deleted in  $\alpha 1$ HS12B. One of the mouse BSAP sites (BSAP2) is not conserved, but most residues in a second mouse BSAP binding site (BSAP1) are

maintained in the human  $\alpha 2$  HS12 enhancer. The murine  $\alpha$ P site, which binds to an ETS-related transcription factor which augments enhancer activity (17), is not conserved in the human sequences.

The DNase I hypersensitive sites lying <3 kb downstream from the membrane exons of C $\alpha$ 1 and C $\alpha$ 2, roughly in the position of the weak enhancer reported by Matthias and Baltimore (11) and here designated HS3A, were found to contain sequences which are 74% identical to the murine HS3 over a 200 bp core segment. The two human HS3 segments are identical in the 326 bp shown (Fig. 5 B), and lie in the same orientation as HS3A. We have assumed that the correct orientation of the murine HS3A sequence is that described by Chauveau and Cogné (13). This orientation is opposite to that of the murine HS3B, which lies downstream from HS12 in the mouse, as described by Madisen and Groudine (12). Of the enhancer/HS sites downstream of murine C $\alpha$ , HS3 is the least well investigated for functional motifs, in part because of its weak enhancer activity. Independent sequence analysis of the murine HS3A and HS3B regions detected several similarities to octamer motifs, AP1 sites, and consensus E box motifs (CANNTG). The AP1 site identified in Fig. 4 is a precise match to the consensus AP1 binding motif TGANTCA (40) in the two human and two mouse HS3 sequences, and the murine sequence has been shown to bind to c-jun and c-fos in vitro (Neurath, M., personal communication). Similarly, several of the E box consensus motifs in the murine sequence have been shown to bind in vitro to proteins of the HLH family (Neurath, M., personal communication); some of these motifs are conserved in the two human HS3 sequences. The significance of the conserved motifs remains uncertain in the absence of a functional analysis of HS3 sequences.

The DNase I hypersensitivity sites furthest downstream



**Figure 5.** Sequence similarities between human and mouse 3'  $\alpha$  elements. Human-mouse alignments are shown between 3'  $\alpha$  enhancers (HS12, HS3, and HS4), as well as for the X DNase I site, Nucleotide matches between human  $\alpha 1$  and  $\alpha 2$  sequences and between one or both human sequences and mouse are indicated by shading. Core homology regions are indicated by a thick line above the sequences. Boxes denote motifs shown to function in mouse as transcription factor binding sites. For HS12, HS3, and HS4, 50–100 bp of sequence flanking the core homology regions are shown. Mouse sequence numbering is 5' to 3' with regard to the coding strand of the mouse heavy chain locus. Numbering for mouse HS12, HS3A, HS3B, and X segments is according to reference 13 (EMBL/GenBank/DBJ accession numbers X96607 and X96608), while numbering for mouse HS4 is according to reference 12 (EMBL/DBJ/GenBank accession number S74166). (A) HS12 sequences ( $\alpha 2$  sequence inverted). Overlining highlights the striking 135-bp core segment which is 90% homologous between human and mouse. The sequence alignment has been extended downstream from the core to include additional transcription factor motifs which are functional in mouse. Vertical lines indicate the boundaries of the GC-rich 59-bp repeat units. (B) HS3. Comparison of the nearly identical human  $\alpha 1$  and  $\alpha 2$  HS3 sequences with mouse HS3A and HS3B sequences, which are also nearly identical, shows that there is a 200-bp core segment which is 74% homologous between the mouse and human sequences. (C) HS4. Excluding the 25-bp gap containing the mouse HS4 BSAP site, the 145 core HS4 region is 76% homologous between human and mouse. (D) X site. Near the center of a 61-bp segment which has 70% human-mouse homology, there is a 20-bp sequence which matches at 19 positions between humans and mice. In both mice and humans this segment contains a consensus HSE (41, 42). The sequences of the human enhancers and X sites are available from EMBL/GenBank/DBJ under accession numbers AF013718 ( $\alpha 1$ HS3), AF013719 ( $\alpha 2$ HS3), AF013720 ( $\alpha 1$ X), AF013721 ( $\alpha 2$ X), AF013722 ( $\alpha 1$ HS12), AF013723 ( $\alpha 1$ HS12B), AF013724 ( $\alpha 2$ HS12), AF013725 ( $\alpha 1$ HS4), and AF013726 ( $\alpha 2$ HS4).

from human  $\alpha 1$  and  $\alpha 2$  in Fig. 3, which we have designated HS4, are 76% similar to the murine HS4 site over a core 145-bp sequence which spans the three functional motifs demonstrated in murine HS4 (23); see Fig. 5 C. The NF- $\kappa$ B motif and the downstream octamer motif in Fig. 5 C are both precisely conserved; in the murine HS4 these motifs both contribute to functional enhancer activity (23). In contrast, the BSAP site which upregulates murine HS4 enhancer activity in B cells but downregulates it in pre-B cells is completely absent from the human HS4 sequences.

Lying between HS3 and HS12 in both the  $\alpha 1$  and  $\alpha 2$  loci are DNase I hypersensitive sites which are not associated with any of the known enhancer elements, but that do map to the position of a 61-bp segment of 70% mouse-human homology. These conserved regions are provisionally designated X sites in part because of their unknown function, and in part because both the human and mouse segments contain XbaI restriction enzyme sites. In mouse this sequence is duplicated as part of the large inverted repeat centered on HS12, so that one copy lies between HS3A and HS12 while a second copy lies between HS12 and HS3B. A segment of (GA) $_n$  repeats is found near the X

site in the direction of HS12 at an interval of 80 bp for both mouse X sites (13) and an interval of  $\sim 70$  bp for the human (data not shown). Within the 61-bp conserved segment, the most highly conserved sequence is a consensus heat shock element (HSE; 41, 42). An HSE could potentially bind heat shock transcription factors (HSTFs), which are known to activate several heat shock response genes (HSP70, HSP90) in response to cellular stress such as heating (43–45). Fragments containing an X site do not appear to dramatically affect enhancer activity in HS Sultan, but may contribute to regulation through mechanisms not captured in our transient transfection assays.

## Discussion

**Structure of Human 3'  $\alpha$  Regions: Evolutionary Implications.** The enhancers clustered 3' of  $\alpha$  in the mouse IgH locus can activate the upstream genes, functioning as an LCR. In human IgH locus, arrays of enhancers homologous to those 3' of mouse  $\alpha$  are located at two positions within the human IgH locus, 3' of each  $\alpha$  gene. On the basis of sequence homology and conservation of restriction

sites between the two human enhancer arrays, it is apparent that these enhancers lie near the 3' ends of the two duplication units which encompass the  $\gamma 3$ - $\gamma 1$ - $\psi \epsilon$ - $\alpha 1$  and  $\gamma 2$ - $\gamma 4$ - $\epsilon$ - $\alpha 2$  gene clusters (25–27), indicating that the 3'  $\alpha$  enhancer arrays were present in an approximation of the human arrangement preceding the duplication event that gave rise to the present human IgH locus structure. Moreover, the arrangement downstream of both human C $\alpha$  genes is 5'-HS3-HS12-HS4-3', in contrast to the large palindromic structure downstream from mouse C $\alpha$  that contains a 5'-HS3A-HS12-HS3B-HS4-3' arrangement (13). Therefore, although an arrangement containing an HS3 enhancer proximal to the C $\alpha$  membrane exon and an HS4 enhancer farther downstream would seem to have been present in the common ancestor of rodents and primates, the mouse HS3A-HS12-HS3B palindrome probably arose after the primate-rodent divergence. Finally, there is a major structural difference between the 3' $\alpha 1$  and 3' $\alpha 2$  enhancer arrays; namely, that a DNA segment containing HS12 is inverted between the two loci. Using probes containing the 135-bp human HS12 core, it should now be possible to examine DNA from a number of primates for inversion of 3' $\alpha 2$  HS12 relative to 3' $\alpha 1$  HS12; such data may indicate when in evolution the inversion event occurred, and which orientation was present initially in the locus. What caused this inversion? Interestingly, the single mouse HS12 lies in opposite orientation from the rat HS12, and both are flanked by inverted repeats (13) which are known to mediate inversions in other genomic contexts, e.g., in the iduronate-2-sulfatase gene causing Hunter syndrome (46) and in the factor VIII gene (47). Limited Southern blot experiments have not provided evidence for inverted repeats flanking the human HS12 sequences (data not shown). Some hints about the mechanism of the inversion may be found when the inversion breakpoints are identified and sequenced, work currently in progress in our laboratory.

**HS12 Structure and Function.** The 135-bp HS12 core homology sequence is likely to contain essential motifs important for the strong, late, B cell-specific enhancer activity characteristic of HS12 in mice and humans. Although the function of transcription factor binding sites within the human HS12 core has not yet been demonstrated experimentally, this segment contains sequences nearly identical to the murine AP1, ETS, Oct, and, in  $\alpha 1$ HS12T and  $\alpha 2$ HS12,  $\mu$ E5 motifs, all of which are functional in the mouse HS12 enhancer. However, the high degree of sequence conservation in the HS12 core homology extends beyond the transcription factor-binding sites identified in the mouse enhancer, indicating that there may be additional conserved motifs that have not been characterized in either mice or humans.

Despite the fact that a number of other transcription factor motifs whose function has been demonstrated in the mouse HS12 lie outside the HS12 135-bp core homology and are absent in one or more of the human  $\alpha 1$ HS12T,  $\alpha 1$ HS12B, and  $\alpha 2$ HS12 enhancers that we have studied, these enhancers all show roughly equivalent activities. This result suggests that elements missing from these enhancers

are not essential for enhancer function in HS Sultan. These inconsistently conserved elements include human sequences corresponding to  $\mu$ E5 and NF- $\kappa$ B sites (absent from  $\alpha 1$ HS12B) and the BSAP2 site (absent from both  $\alpha 1$  alleles). On the other hand, a 1.3-kb  $\alpha 2$  EcoRI-HindIII fragment containing the  $\alpha 2$  HS12 core plus considerable flanking sequence shows a dependence of enhancer activity on orientation of the fragment (Table 2), suggesting that uncharacterized elements beyond the HS12 core may have some inhibitory function.

Outside the human HS12 core are GC-rich 59-bp repeat units which by themselves do not have enhancer activity in the HS Sultan myeloma (EP300, Fig. 4 A), and are not conserved between mice and humans (Fig. 5 A). However, it is possible that these repeats contribute to enhancer activity because they are present in the A2HS12 PCR-generated fragment, which gives significantly higher enhancer activity than we observe in the p300 fragment containing the core homology. Deletions of the 59-bp repeats have given rise to apparent allelic polymorphisms, as evidenced by  $\alpha 1$ HS12T (deletion of the second and third repeats found in  $\alpha 2$ HS12),  $\alpha 1$ HS12 (deletion extending from 28 bp 5' of the first repeat through the third repeat), and other alleles (Harindranath, N., unpublished results).

**HS3 and HS4.** The other two enhancer components of the mouse 3'  $\alpha$  LCR, HS3 and HS4, are weaker enhancers than HS12, but nonetheless are essential for locus control activity (12). Although these elements are less well characterized than the HS12 enhancer, the existing data indicate general human-mouse similarity of the HS3 and HS4 elements, with some notable differences.

In the mouse system, the HS3A element assayed in CAT reporter gene constructs driven by c-fos or thymidine kinase promoters (11) showed weak enhancer activity, although the nearly identical HS3B enhancer showed substantial activity in certain constructs with other promoters tested by another laboratory (12). These disparate results resemble our data on the human  $\alpha 1$  and  $\alpha 2$  HS3 elements in that single copies and dimers of human HS3 generally gave very low enhancer activity, except in the mouse S194 myeloma in which the same constructs gave substantial enhancer activity comparable to that of HS12 (Table 1). Taken together, these data suggest that HS3, though typically the weakest of the 3'  $\alpha$  enhancers, contains uncharacterized motifs that in some cells and/or in combination with certain promoters, can mediate a strong enhancer function.

HS4 is the most downstream 3'  $\alpha$  enhancer in both mice and humans, and shows activity intermediate between that of HS3 and HS12. The HS4 enhancer data in the mouse (12, 48), as well as our data on the human  $\alpha 1$  and  $\alpha 2$  HS4 elements, demonstrate that HS4 is active from the early stages of the B cell lineage onward (Table 2), and thus is qualitatively different from HS3 and HS12. In mouse HS4, there is a binding site for the BSAP, which is expressed in the early B cell lineage. However, in the human  $\alpha 1$  and  $\alpha 2$  HS4 enhancers, the BSAP site is deleted, indicating that BSAP binding is not an essential feature for HS4 activation

in human pre-B cells. The human HS4 is inactive in the 18-81 mouse pre-B cell line, which was reported to support the activity of mouse HS4 (12). The significance of this difference is not clear; it could be related to the BSAP site deletion in the human HS4, or perhaps to other differences between the mouse and human HS4 sequences.

**X Site.** The DNase I X sites may represent novel control elements that function together with the HS3, HS12, and HS4 enhancers to activate the IgH locus. Although the significance of the conserved HSE motif is unclear, binding of HSTF protein to an HSE has been shown to be critical for maintaining the DNase I hypersensitivity of the yeast HSC82 gene promoter (49). Heat shock activation of the *Drosophila* HSP70 gene promoter results from binding of HSTF to HSE sites after accessibility of the HSEs has been established by binding of the GAGA protein to adjacent (CA)<sub>n</sub> repeats (50). This demonstrated interaction between HSEs and (CA)<sub>n</sub> motifs suggests that the location of a (CA)<sub>n</sub> repeat region 70–80 bp away from the X site HSE may be of some significance. Furthermore, in the context of IgH gene regulation, it is of interest to note that HSE motifs have been shown to respond to IL-2 and IL-4 (51).

**Potential Locus Control Region.** In the mouse, it has been demonstrated that when HS3, HS12, and HS4 are linked together in a construct containing the *c-myc* gene and stably transfected into the Raji human B cell line, the *c-myc* gene is transcribed independent of integration site (12). This observation suggests that the HS1234 combination confers LCR activity, although LCRs have more typically been described based on position-independent transcription of mouse transgenes rather than genes transfected into a cell line. Because the regions 3' of the human C $\alpha$ 1 and C $\alpha$ 2 genes contain similar HS3, HS12, and HS4 elements that function as enhancers, it is reasonable to hypothesize that these elements also function together in the human system as LCRs. The different arrangement of 3' $\alpha$  enhancers in mice and humans (HS3A-HS12-HS3B-HS4 versus HS3-HS12-HS4) may cause some functional differences in these control regions. Moreover the distance between 3' $\alpha$  enhancers also differs between mice and humans, with the mouse

enhancer complex spanning a 30-kb region (13), whereas both the human 3' $\alpha$ 1 and 3' $\alpha$ 2 enhancers span ~15 kb.

Our finding that arrays of enhancers homologous to those in the mouse 3' $\alpha$  LCR lie downstream of both human C $\alpha$  genes raises the possibility that differences in the activation of each human  $\gamma$ - $\gamma$ - $\epsilon$ - $\alpha$  duplication unit result from differences between the putative 3' $\alpha$ 1 and 3' $\alpha$ 2 LCRs. Even though sequence comparison shows that there is near identity between homologous enhancer elements in the  $\alpha$ 1 versus  $\alpha$ 2 locus (Fig. 5), transcription and expression of the upstream heavy chain duplication unit ( $\gamma$ 3- $\gamma$ 1- $\psi$  $\epsilon$ - $\alpha$ 1) is greatly elevated relative to the downstream unit ( $\gamma$ 2- $\gamma$ 4- $\epsilon$ - $\alpha$ 2; reference 2). This difference could result from the fact that the 3' $\alpha$ 2 HS12 element is inverted relative to the 3' $\alpha$ 1 HS12, and is also at a greater distance from HS3 than in the  $\alpha$ 1 locus, possibly reducing synergistic interactions between HS3 and HS12. Alternatively, it may be that only the 3' $\alpha$ 1 enhancers are activated early in B cell development, possibly falling under the influence of the upstream E $\mu$  enhancer, which itself can function as an LCR (52, 53). In this model, the E $\mu$  and 3' $\alpha$ 1 enhancers together would activate a large domain encompassing the first duplication unit, whereas the second duplication unit and the 3' $\alpha$ 2 enhancers would fall outside this combined domain. Thus activation of the second duplication unit would depend solely on the 3' $\alpha$ 2 enhancers, and expression of genes in this unit might therefore be reduced. Clarification of the basis for this difference will have to await experiments that involve specific deletion of either the 3' $\alpha$ 1 or 3' $\alpha$ 2 enhancers, as well as studies identifying matrix attachment sites and chromatin insulator elements that define domains within the human IgH region (52–54).

In the work presented here, we have laid the foundation for experimental studies on the activation of the human IgH gene transcription, as well as the regulation of isotype switching. In addition, knowledge of the action of these enhancers on distant constant region genes should contribute to a general understanding of the mechanisms underlying activation of large gene clusters.

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*Note added in proof.* While this manuscript was under review, related investigations by two other laboratories came to our attention. Chen, C., and B.K. Birstein (1997. *J. Immunol.* 159:1310–1318.) have described the HS12 enhancers from the  $\alpha$ 1 and  $\alpha$ 2 loci; and recently others have characterized the HS3 and HS12 enhancers from the  $\alpha$ 1 locus (M. Cogné, personal communication).

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# Introduction of a $\mu$ Immunoglobulin Gene into the Mouse Germ Line: Specific Expression in Lymphoid Cells and Synthesis of Functional Antibody

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## Summary

A functionally rearranged  $\mu$  heavy chain immunoglobulin (Ig) gene was introduced into the germ line of mice. The  $\mu$  gene encodes a polypeptide which, combined with  $\lambda$ 1 light chains, shows a specificity for binding the hapten NP. Four transgenic mice harboring 20–140 copies of the foreign  $\mu$  gene expressed the gene specifically in spleen, lymph node, and thymus at a high level. Purified surface Ig-positive B cells, Lyt 2-positive mature T cells, and thymocytes transcribed the foreign  $\mu$  gene at a similarly high level, suggesting that control of Ig gene rearrangement might be the only mechanism that determines the specificity of heavy chain gene expression within the lymphoid cell lineage. No transcription of the foreign  $\mu$  gene was detected in nonlymphoid tissues with the exception of the heart which expressed the gene at a low level. The transgenic mice had up to 400-fold elevated serum levels of NP binding antibody, which contained a heavy chain with the characteristics of the foreign  $\mu$  gene. The serum levels of endogenous heavy and light chains in transgenic mice appeared to be the same as in normal mice.

## Introduction

Regulation of immunoglobulin (Ig) gene expression is known to occur on different levels. Ig genes are assembled from multiple DNA segments at early stages of B lymphocyte differentiation (reviewed by Tonegawa, 1983). The assembled genes can be transcribed  $10^4$ -fold more actively than their unrearranged counterparts, implying that DNA rearrangement is required for maximal gene transcription (Mather and Perry, 1981). Unrearranged or incompletely rearranged Ig genes, however, although apparently silent in fibroblast cells, are transcribed at a low, but detectable, level in B and even T cells indicating that lymphoid cells contain specific factors that can activate transcription of Ig genes independently of DNA rearrangement (Kemp et al., 1980; Van Ness et al., 1981; Alt et al., 1982). Transcription of the rearranged Ig genes is further regulated during the later stages of the B cell lineage: a

dramatic increase in the level of Ig gene transcription occurs when B cells encounter antigen and differentiate into plasma cells in which Ig mRNA accounts for about 5% of the total mRNA (Schibler et al., 1978).

Some of the underlying mechanisms that allow for regulation of Ig gene expression have begun to be elucidated through experiments in which cloned, rearranged Ig genes are transfected into cultured lymphoid or nonlymphoid cells. Such genes are efficiently expressed when introduced into B lymphoid cell lines, but fail to be expressed when transfected into fibroblast cells (Rice and Baltimore, 1982; Gillies et al., 1983; Neuberger, 1983; Queen and Baltimore, 1983; Stafford and Queen, 1983). This implies that the *trans*-acting factors present in the cells of the B cell lineage may interact with *cis*-acting DNA sequence elements closely linked to the transfected Ig genes, to activate their transcription in a tissue-specific fashion. Gene transfer experiments using in vitro modified Ig genes have identified certain *cis*-acting regulatory elements, such as "enhancer" sequences, associated with heavy chain and kappa light chain genes, which appear able to stimulate transcription in B cells but not in fibroblasts (Gillies et al., 1983; Banerji et al., 1983; Queen and Baltimore, 1983; Picard and Schaffner, 1984).

To gain a more complete picture of the factors responsible for Ig gene regulation during B cell differentiation a system is required that allows detailed analysis of the developmental regulation and tissue specificity of introduced genes. To this end, we have adopted the approach of gene transfer into the mouse germ line. Another rationale for undertaking such experiments is that they would test models of control of Ig rearrangement (Alt et al., 1980b, 1981; Lewis et al., 1982).

Cloned genes can be transferred into the mouse germ line by microinjection into the pronuclei of mouse zygotes (Gordon et al., 1980; Costantini and Lacy, 1981; Wagner et al., 1981; Brinster et al., 1981). Such microinjected genes frequently integrate into chromosomes, are retained throughout development, and are transmitted to offspring as Mendelian traits. A number of different cloned genes have been transferred into the mouse germ line in this manner. Several microinjected foreign genes have shown a tendency to be expressed in the correct tissues (Palmiter et al., 1982; McKnight et al., 1983). Recently Brinster et al. (1983) demonstrated that a microinjected rearranged mouse  $\kappa$  light chain gene is efficiently expressed in spleen but not in liver. Thus it appears that in some cases *cis*-acting tissue-specific regulatory mechanisms continue to function after gene transfer into the mouse germ line. We hoped that this approach might also be exploited in the study of the regulation of Ig heavy chain gene expression.

In this paper we describe an analysis of five transgenic mouse lines carrying a cloned, rearranged mouse heavy chain gene. We have examined the transcription of the foreign Ig gene in various tissues of several of these mice, and the data show that the gene is transcribed specifically in B and T lymphoid cells. In addition, we present evidence that four of the five mice synthesize foreign Ig heavy chain

polypeptides which combined with endogenous Ig light chains to form functional antigen-specific antibody. We also find that expression of the microinjected  $\mu$  gene does not appear to affect the serum level of endogenous Ig heavy chains.

## Results

The variable region of the heavy chain gene we chose for introduction into the murine germ line contains a member of the NP gene family of  $V_H$  gene segments which is expressed in the BALB/c hybridoma 17.2.25 (White-Scharf and Imanishi-Kari, 1982; Loh et al., 1983). We chose this gene because it encodes a polypeptide with a well-characterized specificity for binding the hapten 4-hydroxy-3-nitrophenyl (NP) (Imanishi-Kari and Makela, 1974; Karjalainen, 1980). The 17.2.25 heavy chain produces NP binding antibody when it combines with  $\lambda 1$  light chains (White-Scharf and Imanishi-Kari, 1982). Because about 5% of the mature B cells in mice contain functional  $\lambda 1$  light chains deriving their specificity from a single  $V_L$  gene segment (Tonegawa et al., 1978; Selsing et al., 1982), we hoped to monitor the expression of the NP heavy chain gene and the synthesis of a functional polypeptide by measuring the level of NP binding antibody in the serum of transgenic mice. In addition, the immune response against NP in mice is strain-specific (White-Scharf and Imanishi-Kari, 1981, 1982). BALB/c and C57BL/6 mice possess two related, but distinct, sets of dominant NP-specific  $V_H$  gene segments, termed  $V_HNP^a$  for BALB/c mice and  $V_HNP^b$  for C57BL/6 mice. The  $V_H$  gene segments of both sets have been cloned, sequenced and their structural differences have been characterized (Bothwell et al., 1981; Loh et al.,

1983). For the gene transfer experiment we used C57BL/6 mouse embryos as recipients because we expected that the microinjected  $V_HNP^a$  (17.2.25) gene could then be distinguished from the corresponding endogenous  $NP^b$  gene copies at the DNA, RNA, and protein levels.

In the 17.2.25 hybridoma, the rearranged  $V_H$  gene segment is linked to a  $\gamma 1$  constant region. We wanted to introduce a  $\mu$  gene into the germ line of mice because the  $\mu$  isotype is activated first in B cell differentiation. For that reason we used a DNA clone, called pLV2, in which the rearranged  $V_H$  17.2.25 region had been fused to the genomic  $\mu$  gene segment (D. Loh and C. Queen, unpublished data). We isolated the  $\mu$  gene from this clone and inserted the gene into a pBR322 derived vector (Figure 1). The  $\mu$  gene included 2 kb of DNA sequence 5' to the variable region and the entire constant region containing both the secreted and membrane-bound 3' exons (Figure 1). A modified mouse H4 histone gene in the construct was not utilized in the present experiments, but will serve as an internal control gene in future studies. This pBR322-based plasmid DNA construct will be subsequently referred to as  $p\mu$ .

## Transfer of $p\mu$ DNA into Mice

Approximately 50 molecules of  $p\mu$  DNA, linearized with Sal I, was microinjected into the male pronucleus of fertilized C57BL/6 mouse eggs, as previously described (Costantini and Lacy, 1982). A total of 284 microinjected eggs were transferred into the oviducts of nine pseudopregnant (CBA/J  $\times$  C57BL/6J) F<sub>1</sub> female mice, either shortly after microinjection or after culture in vitro to the two-cell stage. Eight of the nine recipient females became pregnant, 23 of the embryos in these mice developed to term, and 13 of these survived to weaning.

When each of the 13 surviving mice was approximately four weeks old, a segment of tail was removed and DNA was extracted and analyzed for the presence of the microinjected gene. Tail DNA from five of the mice was found to contain the foreign gene. DNA samples were digested with Pst I (see Figure 1), fractionated by gel electrophoresis, transferred to nitrocellulose paper and probed with <sup>32</sup>P-labeled pBR322 DNA; Ig gene sequences were not included in the probe because these would cross-hybridize with endogenous mouse Ig genes. Three of the first eight mice that were screened contained DNA sequences hybridizing with pBR322 DNA (Figure 2). The intense 2.9 kb band in each positive lane (c, e, and h) corresponds with an expected Pst I fragment of  $p\mu$  DNA (see Figure 1). In addition, each of the DNAs of the three positive mice—M52, M54, and M57—showed a band at 0.85 kb. This band corresponds in size to the other pBR322-containing Pst I fragment of circular  $p\mu$  DNA, but is about 100 bp larger than the 0.75 kb Pst I-Sal I fragment generated by Pst I digestion of the Sal I-linearized  $p\mu$  DNA used for microinjection (Figure 2, lane j). The presence of the 0.85 kb fragment in the DNAs of the positive mice suggested that the ends of the linear DNA molecules became joined in a head-to-tail orientation after microinjection. Eco RI

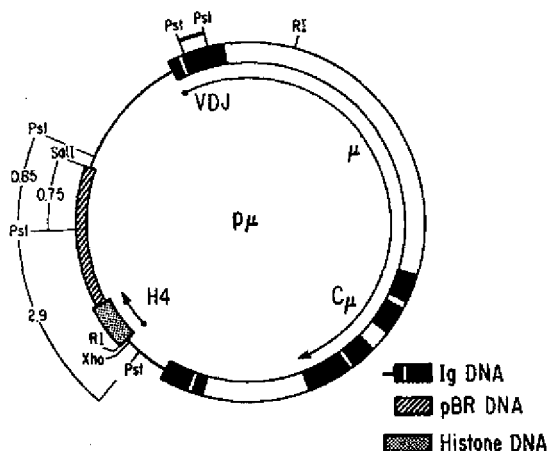


Figure 1. Scheme of the  $p\mu$  Plasmid DNA

The circular map of the plasmid DNA is shown. The  $\mu$  gene is depicted as closed (exons), open (introns), and hatched (noncoding sequences) bars; the 5' and 3' flanking regions of the  $\mu$  gene are represented as lines. pBR322 sequences and H4 gene sequences are drawn hatched or dotted, respectively. Restriction sites used for the DNA analysis of the transgenic mice are indicated. The sizes of the Pst I-DNA fragments that hybridize with pBR322 sequences are denoted.

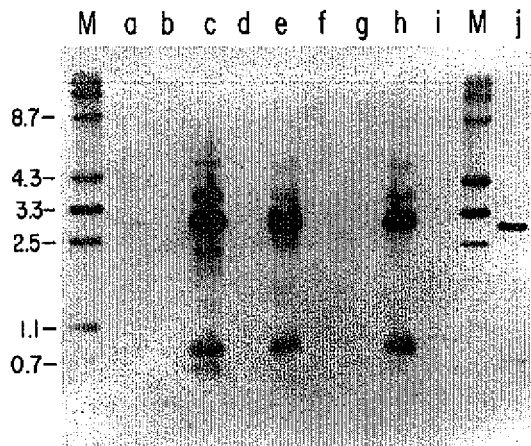


Figure 2. Detection of  $p\mu$  Sequences in Mouse DNAs

Mouse tail DNAs were digested with Pst I, separated by electrophoresis through a 0.7% agarose gel, transferred to nitrocellulose and hybridized with  $^{32}$ P-labeled plasmid pBR322 DNA. Lanes M,  $^{32}$ P-labeled molecular weight markers with the sizes indicated. Lanes a and j, 30 and 300 pg, respectively, of plasmid  $p\mu$  DNA digested with Pst I and Sal I. Lanes b through i, approximately 10  $\mu$ g of tail DNA from mice M51 through M58. The three mice containing  $p\mu$  sequences are M52, M54, and M57.

digestion of the transgenic mouse DNAs confirmed this interpretation: in each case digestion with this enzyme produced a 6 kb fragment that hybridized to pBR322 DNA (data not shown). This fragment is predicted from the circular map of  $p\mu$  DNA (Figure 1). When undigested mouse DNAs were similarly analyzed, the pBR322 sequences migrated with high molecular weight DNA (data not shown). These data, together with the pattern of germ line transmission observed (see below), suggest that the injected  $p\mu$  DNA has integrated into the mouse genome in tandem arrays, as has been frequently observed with other microinjected genes (Costantini and Lacy, 1981; Brinster et al., 1981). The same DNA analysis was applied to the other five mice, two of which, M94 and M95, were also found to contain the microinjected  $\mu$  gene (data not shown).

The number of copies of  $p\mu$  DNA in the genome of each transgenic mouse was estimated by preparing dot blots with varying amounts of the mouse DNAs and of cloned  $p\mu$  DNA, hybridizing with  $^{32}$ P-labeled pBR322 DNA and quantitating the amount of hybridization by liquid scintillation counting. The number of gene copies per diploid mouse genome ranged from about 20 to 140 (Table 1).

The five transgenic mice were mated with normal C57BL/6 mice and tail DNAs from progeny were tested by dot blot hybridization to determine which had inherited the foreign  $p\mu$  sequences. Twenty-seven out of 61 progeny from the male mouse M54, and 18 out of 40 progeny from male M95 contained DNA sequences that hybridized with the pBR322 probe. This frequency of transmission to progeny is consistent with the hypothesis that the foreign DNA was integrated at one site in the germ line DNA of

Table 1.  $p\mu$  DNA and Serum Immunoglobulin Levels in Transgenic Mice

Mouse	$p\mu$ DNA Copy Number	Serum NIP Binding ( $\mu$ g/ml)		Heavy Chain Isotypes	
		$\lambda$	$\kappa$	$\mu$	$\gamma$
M52	140	175.0	459	0.8	1.0
M54	30	91.0	432	0.9	0.9
M57	80	50.0	595	1.4	1.2
M94	68	0.8	54	1.2	1.1
M95	17	6.5	173	0.7	1.0
BL/6	0	0.5	108	1.0	1.0

DNA copy numbers of the  $\mu$  NP<sup>a</sup> gene per diploid genome were determined by quantitative dot blots. Transgenic mouse serum levels of NIP binding antibody with lambda ( $\lambda$ ) or kappa ( $\kappa$ ) light chains are expressed in  $\mu$ g/ml. Relative serum levels of IgM ( $\mu$ ) or IgG ( $\gamma$ ) were determined by competitive radioimmunoassays and normalized to the value for C57BL/6 (BL/6).

these animals. Female M52 has so far transmitted the  $p\mu$  sequences to 12 out of 15 progeny, which might reflect the presence of the foreign DNA on more than one chromosome. Female M57 did not produce any live offspring, and died at seven months of age. Female M94 has not transmitted the gene to any of her 26 offspring tested thus far, suggesting the possibility of extreme mosaicism. A mosaic distribution of the microinjected genes has been observed occasionally by others (Costantini and Lacy, 1981; Wagner et al., 1983; Palmiter et al., 1984).

#### Transcription of the Microinjected $\mu$ Gene

Spleen and liver tissue was obtained from transgenic mice M52, M54, and M57 by partial splenectomy and hepatectomy. Total RNA was isolated, size-fractionated by electrophoresis, transferred to nitrocellulose paper, and analyzed for the presence of transcripts from the microinjected sequences using a specific  $^{32}$ P-labeled DNA probe. The probe was a cloned 220 bp Pst I fragment of the  $V_H$ 17.2.25 gene segment (see Figure 1). A 2.4 kb transcript corresponding in size to the secreted form of  $\mu$  mRNA was detected in the spleen RNA from the transgenic mice (Figure 3, lanes f-h). The 2.7 kb membrane-bound form of the  $\mu$  mRNA could not be detected. This is in agreement with the previous demonstration that total spleen RNA contains only minor amounts of membrane-bound form of heavy chain transcripts (Alt et al., 1980a). The specific probe did not hybridize with spleen RNA from a normal C57BL/6 mouse (Figure 3, lane e), proving that the 2.4 kb transcript detected in the spleen RNA of the transgenic mice was derived from the introduced  $\mu$  gene. To demonstrate the specificity of the DNA probe for  $V_H$ 17.2.25 sequences under our hybridization conditions we hybridized the labeled probe to total RNA from myeloma MPC11 (lane i). Although the expressed  $V_H$  gene segment in MPC11 cells shares extensive sequence homologies with the  $V_H$ 17.2.25 gene segment (Bothwell et al., 1981; Loh et al., 1983), no cross-hybridization of the heavy chain transcripts of MPC11 with the labeled DNA probe was observed. RNA isolated from the hybridoma 17.2.25 was

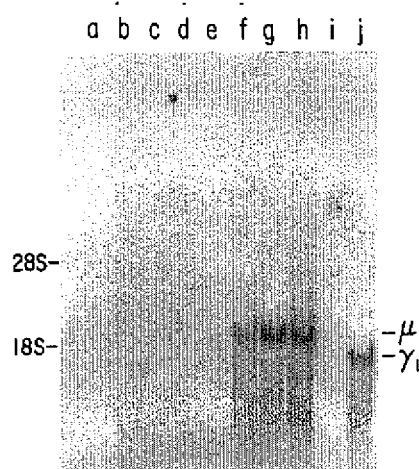


Figure 3. Size Analysis of  $V_H17.2.25$  Specific Transcripts in Liver and Spleen RNA of Transgenic Mice

Ten micrograms of total formaldehyde-treated RNA was separated by electrophoresis through a 1% agarose gel in 6% formaldehyde, transferred to nitrocellulose paper and hybridized with a cloned  $^{32}\text{P}$  nick-translated 220 bp Pst I DNA fragment of the  $V_H17.2.25$  segment. The sizes of the  $V_H17.2.25$  transcripts were calculated from the position of the transcripts on the autoradiogram relative to the positions of 28S and 18S RNA on the ethidium bromide-stained gel. Lanes a-d were loaded with liver RNA, lanes e-h with spleen RNA. Lanes a and e contained RNA from a control C57BL/6 mouse, lanes b and f contained RNA from mouse M52, lanes c and g contained RNA from mouse M54 and lanes d and h contained RNA from mouse M57. Lane i was loaded with RNA from the myeloma MPC11 and lane j contained RNA from the hybridoma 17.2.25.

included as a positive control and revealed the authentic 1.7 kb  $\gamma_1$  mRNA (lane j). No hybridization of the labeled DNA probe could be detected in the lanes (b-d) loaded with liver RNA of the three transgenic mice. By inspection of the rRNA bands in the ethidium-bromide stained gel, the integrity and quantity of the liver RNA appeared to be the same as that of the spleen RNA (data not shown). The absence of specific  $\mu$  transcripts in liver RNA was not due to a mosaic distribution of the introduced DNA in the transgenic mice because we confirmed the presence of the microinjected gene in both tissues of the animals by hybridization to  $^{32}\text{P}$ -labeled pBR322 DNA (data not shown). These results demonstrate that the foreign  $\mu$  gene is transcribed at a high level in the spleen but not in the liver of transgenic mice.

To confirm the tissue-specific expression of the transferred gene with a more sensitive assay and to examine the site of initiation of transcription we assayed spleen and liver RNA from the three transgenic mice by hybridization to a  $^{32}\text{P}$ -labeled single-stranded DNA probe followed by S1 nuclease digestion and electrophoretic separation (Figure 4B). Spleen RNA of the transgenic mice protected an approximately 57 nucleotide long DNA fragment (Figure 4A, lanes b-d). The same protected fragment was generated by S1 nuclease treatment of hybridized 17.2.25 hybridoma RNA (lane a). Thus the identical initiation site of transcription is used in the introduced gene of the trans-

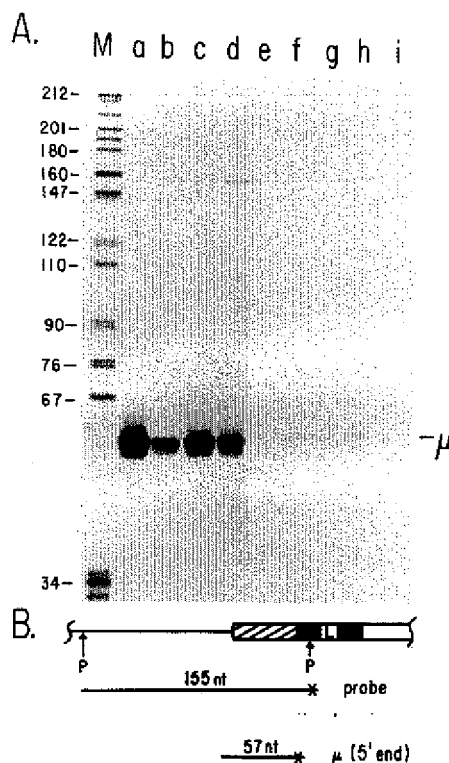


Figure 4. S1 Nuclease Analysis of  $V_H17.2.25$  Specific RNA 5' Ends of Spleen and Liver RNA

(A) Two micrograms of spleen RNA and 20  $\mu\text{g}$  liver RNA from transgenic mice were hybridized to a single-stranded  $^{32}\text{P}$  5' end-labeled DNA probe (see [B] for structure and size of probe) and digested with S1 nuclease as described in Experimental Procedures. The protected fragment was separated by gel electrophoresis and autoradiographed. Four micrograms of 17.2.25 hybridoma RNA was used for S1 nuclease mapping. Lane M,  $^{32}\text{P}$ -labeled Hpa II-digested pBR322 DNA. Lane a, RNA from 17.2.25 hybridoma. Lanes b, c, and d, spleen RNA from transgenic mice M52, M54, and M57, respectively. Lanes f, g, h, liver RNA from mice M52, M54, and M57. Lanes e and i, spleen and liver RNA from normal C57BL/6 mouse. The position of the 57 nucleotide long protected DNA fragment is indicated with  $\mu$ . The bands migrating at 155 nucleotides correspond to RNA protecting the full length of the DNA probe.

(B) Structure of the leader region of the microinjected  $\mu$  gene. The leader sequences (L) are drawn in black; intron sequences are drawn as open, and 5' noncoding sequences are drawn as hatched bars. The structure and size of the DNA probe, which consists of a 155 bp Pvu II (P) DNA fragment, and that of the DNA fragment protected by the 57 5' terminal nucleotides of the specific  $\mu$  RNA are shown. The position of the  $^{32}\text{P}$  label is indicated by a star.

genic mice as in the endogenous gene of the hybridoma. A few specific  $\mu$  transcripts were detected in the liver RNA of the transgenic mice (lanes f-h). The presence of  $\mu$  transcripts in liver was probably due to the blood in this organ because we could decrease the number of specific  $\mu$  transcripts to undetectable levels by perfusion of the transgenic liver prior to DNA isolation (see Figure 5A). No  $V_H17.2.25$  specific sequences were detected in spleen or liver RNA from control C57BL/6 mice (Figure 4A, lanes e and i).

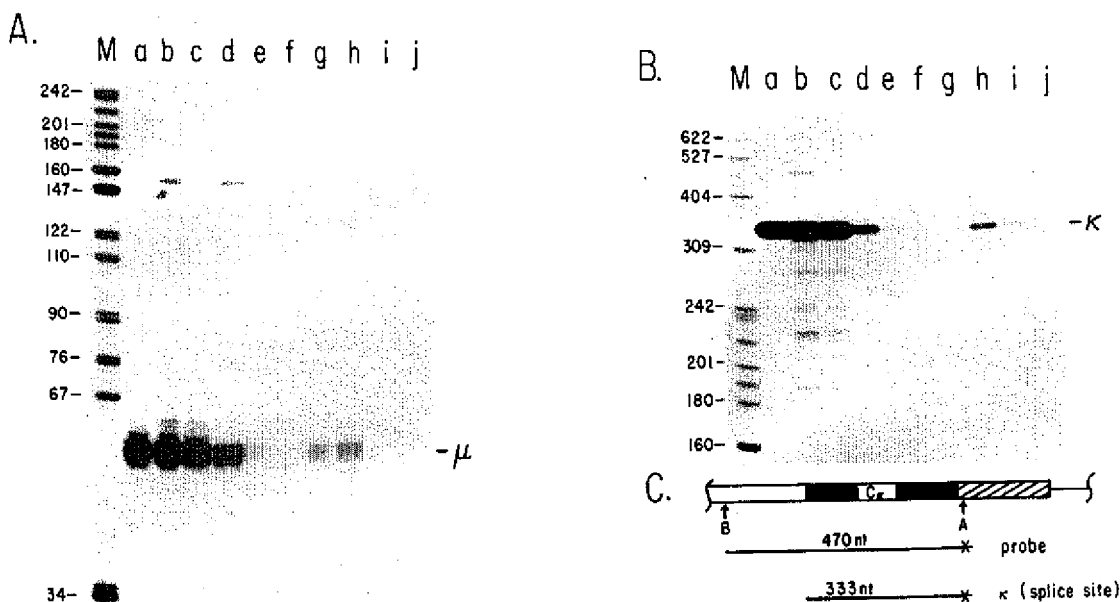


Figure 5. S1 Nuclease Analysis of V<sub>H</sub>17.2.25 Specific and κ Specific Sequences in RNA from Various Tissues of the Transgenic Mouse M54-6  
(A) Analysis of the 5' ends of V<sub>H</sub>17.2.25 specific μ transcripts. Ten micrograms of total RNA from the tissues was hybridized to the <sup>32</sup>P-labeled 155 nucleotide long Pvu II DNA fragment shown in Figure 4B. Four micrograms of 17.2.25 hybridoma RNA was used for hybridization. The position of the DNA fragments protected by the 5' terminal sequences of V<sub>H</sub>17.2.25 transcripts is marked with μ. Lane M, size marker (pBR322 DNA cleaved with Hpa II). Lane a, 17.2.25 hybridoma RNA. Lanes b through j, RNA from lymph nodes, spleen, thymus, kidney, brain, heart, lung, liver, fibroblasts.  
(B) Analysis of κ transcripts. Five micrograms of total RNA was hybridized to the splice probe shown in Figure 5C. The position of the protected fragment is indicated with κ. Lane M, size marker. Lane a, RNA from myeloma MPC11 cells. Lanes b through j are numbered as in (A).  
(C) Structure of the κ splice probe. The exon of the C<sub>κ</sub> constant region is depicted as a black bar. Intron and 3' noncoding sequences are drawn as open and hatched bars, respectively. The positions of the Ava II (A) and Bst NI (B) sites, used to generate the DNA probe, are shown.

The relative number of V<sub>H</sub>17.2.25 specific transcripts in the spleens of the transgenic mice M52, M54, and M57 were determined by densitometric scanning of different exposures of the autoradiogram using transcription of the rearranged endogenous V<sub>H</sub>17.2.25 gene in the hybridoma as standard. The number of specific μ transcripts in the spleen of mouse M54 was calculated to be the same as that in the 17.2.25 hybridoma. This hybridoma was determined to contain 2000 V<sub>H</sub>17.2.25 specific RNA molecules per cell (unpublished results). The spleens of mice M52 and M57 accumulated 35% and 85% of the number of specific μ transcripts found in the hybridoma. Mouse M52, with the highest copy number of the introduced gene (140 copies), has a 3-fold lower RNA level in spleen cells compared with mouse M54 containing 30 foreign μ gene copies (see Table 1). Therefore the levels of transcription of the microinjected genes did not correlate with the number of gene copies integrated in the chromosomes.

#### Tissue Specificity of μ Gene Transcription

A more detailed study of the tissue specificity of μ gene expression was carried out using progeny of mice M54 and M95 to avoid the possibility of a mosaic distribution of the injected gene. Various tissues were collected from a six-week-old second generation mouse, M54-6, and total

RNA was isolated. RNA from the tissues was analyzed by S1 nuclease mapping using a 5' end-labeled specific DNA probe (Figure 5A). High levels of specific μ gene transcription were detected in all lymphatic tissues analyzed. Lymph node RNA contained twice as much V<sub>H</sub>17.2.25 specific μ RNA as spleen (Figure 5A, lanes b and c). The level of specific μ gene transcription in thymus was determined to be about 35% of that in spleen (lane d).

The expression of the microinjected μ gene in lymph nodes was so high that any contamination of a tissue with lymph nodes would have resulted in detection of specific μ transcripts. Thymus preparations are known to be easily contaminated with parathymic lymph nodes. Although we attempted to avoid the parathymic nodes in the dissection, to measure B cell contamination we examined transcription of the endogenous κ light chain Ig gene, a gene whose transcription should be restricted to B cells and is active in about 95% of such cells. Because the 5' ends of κ mRNAs differ from one another, we prepared a 5' end-labeled DNA probe which spanned the boundary of the large J/C<sub>κ</sub> intron and the C<sub>κ</sub> exon (Figure 5C). We used this labeled DNA probe for S1 nuclease mapping of the splice site at the 5' end of the C<sub>κ</sub> exon which is common to all κ mRNAs.

RNA from the tissues was hybridized with the "splice

probe," digested with S1 nuclease and the protected fragments were separated by electrophoresis. RNA from spleen and lymph nodes contained similar numbers of  $\kappa$  transcripts (Figure 5B, lanes b and c). As a positive control, RNA from cells of the myeloma MPC11 was used (lane a). The presence of  $\kappa$  gene transcripts in thymus (lane d), determined to be about 12% of the number in spleen, revealed contamination of the thymus preparation with  $\kappa$ -producing cells. This level of contamination with B cells, however, does not account for the high level of 17.2.25 gene expression observed in thymus (compare Figure 5A) indicating that the microinjected gene is probably active in thymocytes.

Nonlymphoid tissues were also analyzed for the presence of V<sub>H</sub>17.2.25 specific  $\mu$  RNA sequences. Heart and lung both contained  $\mu$  transcripts (Figure 5A, lanes g and h) which represented about 5% and 8% of the number of specific  $\mu$  RNA molecules detected in spleen. Kidney RNA contained about 2% of V<sub>H</sub>17.2.25 specific transcripts present in spleen (lane e). No  $\mu$  transcripts were detected in RNA from brain, perfused liver, or primary fibroblasts cells (lanes f, i, and j). To assess the question of whether the specific  $\mu$  transcripts present in heart, lung, and kidney were due to transcription of the microinjected gene in these tissues or due to contaminating lymphoid cells in the blood of the tissues we again measured  $\kappa$  transcripts in the RNA preparation from these tissues. Transcripts containing C<sub>1</sub> sequences were found in the RNA from lung tissue, at about 5% of the level in spleen (Figure 5B, lane h). The extent of B cell contamination of lung thus could account for the  $\mu$  transcripts found, suggesting that the microinjected gene was not transcriptionally active in this tissue. The presence of some  $\kappa$  transcripts in kidney RNA (Fig. 5B, lane e) indicated that kidney was contaminated with B cells and, again, that the microinjected gene was probably not transcribed. Heart was the only nonlymphatic tissue of the transgenic mouse M54-6 in which the microinjected gene appeared to be active because virtually no  $\kappa$  transcripts could be detected in RNA from that tissue (Figure 5B, lane g).

This tissue distribution of specific  $\mu$  transcripts was confirmed by analysis of another second generation mouse, M54-3, and similar results were also obtained with a progeny from transgenic mouse M95 (data not shown).

#### Cell-Type Specificity of Transcription

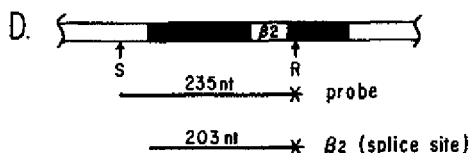
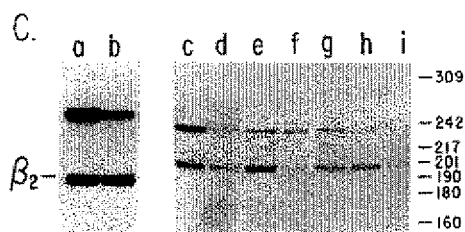
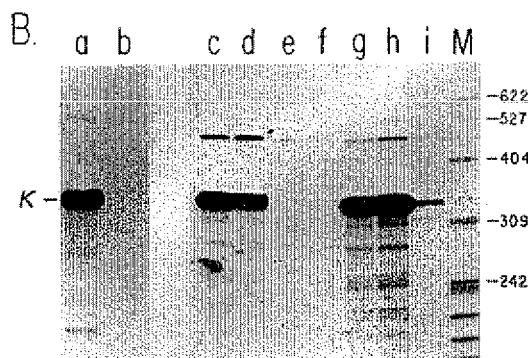
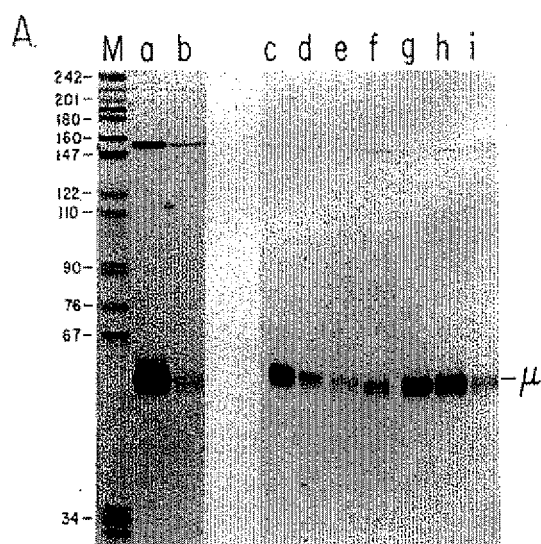
To investigate the expression of the microinjected  $\mu$  gene in individual lymphoid and nonlymphoid hematopoietic cell types, a number of fractionation procedures were used. To study macrophages, which belong to the myeloid lineage, peritoneal cells were isolated from a second generation mouse, M54-48, previously injected with thioglycolate to increase the number of macrophages (Gallily and Feldman, 1967). The cells were incubated in a tissue culture plate to allow macrophages to attach. Phagocytosis of latex particles was used to show that virtually all of the adherent cells were macrophages. We isolated total RNA from the cells and assayed for the presence of V<sub>H</sub>17.2.25

sequences by S1 nuclease mapping of the specific mRNA 5' ends. Very few specific  $\mu$  transcripts were detected in the RNA from macrophages (Figure 6A, lane b), about 2% of that found in total lymph node RNA from the same animal (lane a). We could not detect any  $\kappa$  transcripts in the RNA from macrophages (Figure 6B, lane b) indicating that the low level of specific  $\mu$  transcripts in macrophages was not due to contaminating B cells.

For the purification of B and T cells we isolated spleen and mesenteric lymph nodes from second generation transgenic mice M54-62 and M54-64. Cell suspensions were prepared and surface Ig-positive B cells were purified by binding to plastic dishes coated with rabbit anti-mouse Ig. To reduce the background of nonspecific binding of cells because of their F<sub>c</sub> receptor, we used F(ab')<sub>2</sub> fragments of the anti-mouse Ig antibody. The bound cells were washed, RNA was isolated and analyzed by S1 nuclease mapping. V<sub>H</sub>17.2.25 specific  $\mu$  transcripts were detected in the RNA from B cells of spleen and lymph nodes (Figure 6A, lanes c and d), proving that the microinjected  $\mu$  gene is expressed in surface Ig-positive B cells. To purify T cells, cell suspensions from lymph nodes and thymus were first depleted of surface Ig-positive B cells by binding to plastic dishes coated with rabbit anti-mouse Ig. Unbound cells were then transferred to plastic dishes coated with the F(ab')<sub>2</sub> fragment of anti-Lyt 2 antibody. Surface Lyt 2-positive T cells, which should represent 50% of all mature T cells present in lymph nodes and 90% of thymocytes, were bound and RNA was isolated. Analysis of the RNA revealed the presence of specific  $\mu$  transcripts in mature T cells from lymph node and thymus (lanes e and f). The number of specific  $\mu$  transcripts in T cells from lymph nodes was only about 2-fold lower than that in B cells from lymph nodes, indicating that the microinjected gene is almost equally active in mature B and T cells.

These cell populations probably contribute most of the specific  $\mu$  signal seen with total RNA from whole spleen or lymph nodes, because the cells remaining unbound after the above described fractionations did not show a significantly higher level of V<sub>H</sub>17.2.25 specific transcripts (Figure 6A, lanes g-i).

To demonstrate the purity of the B and T cell preparations, we measured  $\kappa$  transcripts in the RNA. Large amounts of  $\kappa$  mRNA were detected in B cell RNA from spleen and lymph nodes, as expected (Figure 6B, lane c and d). Virtually no  $\kappa$  transcripts were found in T cell RNA from lymph node and thymus (lanes e and f), indicating that the T cell preparations were more than 98% free of contaminating B cells. This result verified the observation that Lyt 2-positive T cells from lymph nodes and thymus indeed expressed the microinjected  $\mu$  gene. Because the cells went through some manipulations prior to RNA isolation we wanted to control for the quantity and integrity of the RNA of each cell preparation. We determined the presence of beta-2 microglobulin transcripts in each RNA preparation by S1 nuclease mapping of the splice site of the second exon of the gene (Figure 6D; Parnes and Seidman, 1982). Similar numbers of beta-2 microglobulin



transcripts were detected in B cells of spleen and lymph nodes (Figure 6C, lanes c and d) and in T cells from lymph nodes (lane e). Fewer beta-2 microglobulin transcripts were found in T cells from thymus (lane f).

#### Formation of Functional Antibody by Combination of the Specific $\mu$ Heavy Chain with Endogenous $\lambda$ 1 Light Chains

The synthesis of a functional  $\mu$  17.2.25 polypeptide was monitored by measuring the formation of NP binding antibody, composed of the specific  $\mu$  heavy chains and endogenous  $\lambda$  1 light chains. We tested the sera of the transgenic mice for elevated levels of NP binding antibody by a standard radioimmunoassay using the derivative 5-iodo-NP (NIP). NIP-BSA coated wells were incubated with serial dilutions of serum, and bound immunoglobulins were detected by binding of  $^{125}$ I-labeled rabbit anti-mouse  $\lambda$  antibody. A dramatic increase in the level of NP binding antibody with  $\lambda$  light chain specificity was observed for sera from mice M52, M54, M57, and M95 varying from 5 to 175  $\mu$ g/ml in concentration (Table 1). Normal C57BL/6 mice and negative littermates that did not possess the specific  $\mu$  gene were negative for serum NP binding activity, giving the same low level of 0.5  $\mu$ g/ml. Mouse M94 serum contained only 2-fold more NP binding antibody with  $\lambda$  specificity than normal serum, although the  $\mu$  DNA copy number in its tail was 68 (Table 1). The NP binding antibodies of all transgenic mice have  $\lambda$  1 light chains because a  $^{125}$ I-labeled rat anti-mouse  $\lambda$  1-specific monoclonal antibody, LS136, showed the same level of NP binding as the polyclonal anti-lambda antibody (data not shown).

The serum of transgenic mice showed only a 2- to 3-fold increase in the concentration of  $\kappa$ -specific NP binding antibody, as determined by binding of  $^{125}$ I-labeled rat anti-mouse  $\kappa$  monoclonal antibody, 187.1 (Table 1). The small increase of  $\kappa$ -containing antibody might indicate that the

Figure 6. S1 Nuclease Analysis of  $V_H17.2.25$ ,  $\kappa$ , and Beta-2 Microglobulin Sequences in Total RNA from Transgenic Macrophages, B and T Cells.

(A) Analysis of the 5' ends of  $V_H17.2.25$  specific  $\mu$  transcripts. Two micrograms of total RNA from various cells was hybridized to the  $\mu$  5' end probe shown in Figure 4B. Lane M,  $^{29}$ P-labeled size marker (pBR322 DNA cleaved with Hpa II). Lanes a and b, RNA from lymph nodes and purified macrophages of the transgenic mouse M54-48. Lanes c and d, RNA from surface Ig-positive B cells from spleen and lymph node, respectively. Lanes e and f, RNA from Lyt 2-positive T cells from lymph nodes and thymus. Lanes g through i, RNA from spleen, lymph node, and thymus cells which had been previously depleted of surface Ig- and Lyt 2-positive cells.

(B) Analysis of  $\kappa$  transcripts. Two micrograms of total RNA was hybridized to the  $\kappa$  splice probe shown in Figure 5C. The position of protected fragments is indicated with  $\kappa$ . The lanes are numbered as in (A).

(C) Analysis of beta-2 microglobulin transcripts. Two micrograms of total RNA was used for S1 nuclease mapping with a double-stranded beta-2 splice probe depicted in (D). The lanes are numbered as in (A). The 203 nucleotide long protected fragment is indicated with  $\beta_2$ . The 235 nucleotide long fragment represents the renatured full-length DNA probe.

(D) Structure and size of the beta-2 microglobulin probe. The probe consists of an Eco RI/Sac I DNA fragment, 5' end labeled at the Eco RI site. The probe contains sequences of the second exon of the gene (black bar) and intron sequences (open bar).

$\mu$ NP<sup>a</sup> 17.2.25 heavy chain is capable of forming a functional NP-specific antibody when combined with a few kappa light chains, but the minimal increase is in agreement with the observation that NP-binding antibody produced in the primary immune response in BALB/c mice contains predominantly  $\lambda$ 1 light chains (White-Scharf and Imanishi-Kari, 1981).

The NP-binding antibody in transgenic mice contained  $\mu$  heavy chains because only anti- $\mu$ , and not anti-IgG1, IgG2a, or IgG2b antisera, detected the increased level of NP binding (data not shown). The progeny of M54 and M95 were screened for  $\lambda$ -specific NP binding activity. Positive littermates that inherited DNA sequences were also positive for NP binding activity. In addition, positive littermates had a level of  $\lambda$ -specific NP binding antibody comparable to their transgenic parent. Although we cannot yet rule out that the transgenic mice have shown elevated expression of the endogenous NP<sup>a</sup> gene, it is much more likely that the high constitutive serum level of NP-specific antibody in the transgenic mice results from a combination of the  $\mu$  heavy chain encoded by the microinjected gene with endogenous  $\lambda$ 1 light chains.

The microinjected rearranged heavy chain gene was present from the earliest developmental stages of the transgenic mice. Thus expression of the gene and/or functional antibody might have altered normal immunoglobulin production. As a crude measure we assayed the sera from transgenic mice and their negative littermates for the presence of total  $\mu$  or  $\gamma$  heavy chains, and kappa or lambda light chains, by competitive radioimmunoassay. Transgenic mouse serum competed as well as normal mouse serum with <sup>125</sup>I-labeled IgM, IgG, or kappa antibodies for anti-IgM, IgG, or kappa binding (Table 1). A modest 2-fold increase in lambda containing immunoglobulins was detected in serum from mouse M54. Thus the expression of a microinjected heavy chain gene had no obvious effect on the levels of endogenous serum immunoglobulins.

## Discussion

### High Level of Expression of the Microinjected $\mu$ Gene

The expression of the microinjected rearranged  $\mu$  heavy chain gene in transgenic mice demonstrates that DNA sequences that are required for the transcriptional activation of the gene during B cell ontogeny are located within the introduced DNA construct. The injected Ig gene was found to be expressed at high levels in four out of five transgenic mice. The one exception, mouse M94, had the gene integrated in chromosomal DNA of the tail but revealed only a 2-fold elevation of NP binding antibody in the serum (Table 1). Because this mouse has not transmitted the microinjected gene to any of her 26 progeny so far, we suspect that the lack of NP binding antibody in the serum was due to a mosaic distribution of the injected  $\mu$  gene in spleen and germ line, rather than to a transcriptional inactivity of the gene. A similarly high frequency of

expression has been reported by Brinster et al. (1983) who introduced a rearranged  $\kappa$  light chain gene into the mouse germ line.

These results contrast markedly with those obtained for several other introduced genes, which are expressed in a lower proportion of transgenic mice or at much more variable levels, presumably because of the influence of chromosomal position (Jaenisch et al., 1981; Wagner et al., 1981; Brinster et al., 1981; Lacy et al., 1983; Palminter et al., 1983). The question that arises is why microinjected immunoglobulin genes appear to behave differently, showing only slight variation of expression levels between different transgenic mouse lines. It is possible that the *cis*-acting transcriptional enhancer elements associated with immunoglobulin genes (Gillies et al., 1983; Banerji et al., 1983; Queen and Baltimore, 1983; Picard and Schaffner, 1984) may be capable of counteracting negative chromosomal position effects.

### The Foreign $\mu$ Gene Is Regulated in a Tissue-Specific Fashion in Transgenic Mice

Transgenic mice are ideal for the study of tissue specificity of gene expression because all cells should contain the same number of copies of the introduced gene in the identical chromosomal location. Therefore we can compare directly the level of transcription in one tissue with that in another tissue. Our results demonstrate that expression of the microinjected Ig  $\mu$  gene is tissue specific. High levels of transcription can be detected in lymphoid tissues, while nonlymphoid tissues, with the exception of heart, contain virtually no specific  $\mu$  transcripts.

Because the functionally rearranged foreign heavy chain gene is present in cells that do not produce the specific  $\mu$  transcripts, we can conclude that DNA rearrangement, which occurs only in cells of the B cell lineage, does not solely account for the lymphoid tissue specificity of  $\mu$  gene expression. *Cis*-acting DNA sequences residing in the introduced rearranged  $\mu$  gene must control its selective transcription. A similar conclusion was reached by Brinster et al. (1983), who showed that an introduced rearranged light chain gene was expressed in spleen but not in liver. Both sets of data support DNA transfection experiments which showed that rearranged Ig genes are transcriptionally inactive in fibroblast cells (Gillies et al., 1983; Stafford and Queen, 1983). The heavy chain gene enhancer was demonstrated to be inoperative in fibroblast cells, leading to the hypothesis that this regulatory element determines the tissue specificity of Ig gene expression (Gillies et al., 1983; Banerji et al., 1983). More recent studies in which the heavy chain gene enhancer was replaced with a nonspecific viral enhancer, however, have demonstrated that other regulatory elements are involved in determining tissue specificity of  $\mu$  gene transcription (R. Grosschedl and D. Baltimore, unpublished data).

Although expression of the microinjected  $\mu$  gene was generally restricted to lymphoid tissues, the gene appeared to be transcribed at a low level in heart for reasons that are unclear. One possible explanation is that cardiac mus-

cle cells might contain *trans*-acting factors which can partially substitute for lymphoid cell-specific regulatory factors interacting with regulatory DNA sequences. Heart and hematopoietic cells are derived from common progenitor cells during development and this fact might account for  $\mu$  gene expression in heart. Alternatively, the  $\mu$  genes might be integrated in a chromosomal locus that is activated in heart. This possibility appears unlikely because mouse lines M54 and M95, in which the  $\mu$  genes are presumably integrated at different chromosomal positions, both show  $\mu$  gene transcription in heart.

Macrophages, which are terminally differentiated non-lymphoid hematopoietic cells, showed a very low but detectable level of specific  $\mu$  gene transcription, about 2% of that in spleen. The macrophage preparation was determined to be free of B cells but may have been contaminated with some T cells. Because the  $\mu$  gene is expressed in T cells at high levels, even a minimal contamination could account for the few specific  $\mu$  transcripts. Previous analysis of RNA from a macrophage cell line showed that the endogenous  $\mu$  genes seem to be inactive (Kemp et al., 1980) and our result is consistent with this observation. In contrast, tumor cells representing early stages of the myeloid lineage contain aberrant or "sterile"  $\mu$  transcripts derived from the unrearranged  $\mu$  constant region at a level similar to that found in B cell lymphomas (Kemp et al., 1980). This implies that these immature myeloid cells contain factors that can interact with *cis*-acting elements associated with the heavy chain genes. It remains to be established whether in transgenic mice, cells representing earlier stages of the myeloid lineage transcribe the microinjected  $\mu$  gene at a higher level than terminally differentiated macrophages.

#### The Rearranged Gene is Expressed in Both B and T Cells

The most interesting result we obtained concerning expression of the foreign  $\mu$  gene in transgenic mice was the observation that the gene is almost equally active in purified B and T cells. Surface Ig-positive B cells of mouse M54 synthesize as many  $V_H17.2.25$  specific transcripts as hybridoma 17.2.25 cells. The level of transcription of the microinjected  $\mu$  gene in Lyt 2-positive T cells is only 2-fold lower than that in B cells. This result implies that T cells contain probably all *trans*-acting factors required for high level expression of rearranged Ig heavy chain genes. Previous analysis of RNA from various T cell lines (Kemp et al., 1980; Alt et al., 1982) and whole thymus (Alt et al., 1982) revealed the presence of "sterile"  $\mu$  transcripts. The synthesis of such sterile  $\mu$  transcripts might reflect an active enhancer element because in some cases the 5' ends of sterile  $\mu$  transcripts have been mapped to a pseudopromoter (Nelson et al., 1983) located close to the enhancer (Gillies et al., 1983; Banerji et al., 1983). From our data we can conclude that not only the enhancer but also all other *cis*-acting elements required for high level  $\mu$  gene transcription are active and operate properly in Lyt 2-positive T cells.

The high level expression of the rearranged  $\mu$  gene in T cells contrasts with the normally exclusive pattern of B and T cell receptor gene expression. In normal development, functional rearrangement of the B cell receptor genes (or Ig genes) is restricted to B lymphocytes whereas rearrangement of the T cell receptor genes occurs in T cells (Tonegawa, 1983; Hedrick et al., 1984a, 1984b; Yanagi et al., 1984). Our experiments suggest that control of gene rearrangement may be the only mechanism that determines the specificity of heavy chain expression within the lymphoid cell lineage. Similarly, the cell specificity of T cell receptor expression may also be controlled at the level of DNA rearrangement. It will be very interesting to learn whether genes for B and T cell receptors utilize common *trans*-acting factors for transcriptional regulation. In contrast, the tissue-specific expression of heavy chain genes in lymphoid as opposed to nonlymphoid cells does not depend on the tissue specificity of DNA rearrangement, but is determined by the interaction of lymphoid-specific factors with *cis*-acting regulatory elements.

#### Expression of the Microinjected $\mu$ Gene Does Not Affect the Serum Levels of Endogenous Immunoglobulins

Expression of the foreign  $\mu$  gene in B cells of transgenic mice generated NP binding antibody when the specific heavy chain associated with endogenous  $\lambda 1$  light chains. The serum levels of NP binding antibody in transgenic mice was stimulated up to 400-fold over that in normal C57BL/6 mice (Table 1). This assay probably underestimates the number of specific  $\mu$  heavy chains synthesized, because we monitor only those that have associated with endogenous  $\lambda 1$  light chains.

All transgenic mice had normal serum levels of the endogenous immunoglobulins, suggesting that expression of the foreign  $\mu$  gene did not completely prevent rearrangement and expression of endogenous Ig genes. The mechanism which ensures that only a single heavy chain gene and a single light chain gene will be functionally rearranged during B cell differentiation ("allelic exclusion") may involve a feedback mechanism in which the synthesis of an Ig heavy or light chain polypeptide blocks any further rearrangements of heavy or light chain genes, respectively (Alt et al., 1980b, 1981; Coleclough et al., 1981). We do not yet know whether transgenic B cells express simultaneously the foreign  $\mu$  gene and endogenous heavy chain genes but the transgenic mice offer us the opportunity to study this aspect of immunoregulation in detail.

#### Experimental Procedures

##### Construction of $p\mu$

pLV2 DNA, which has an Eco RI DNA fragment containing the functionally rearranged  $V_H D_{JH}$  gene segment of the hybridoma 17.2.25 and flanking sequences linked to the genomic Eco RI/Xho I DNA fragment containing the  $C_H$  constant region (D. Loh and C. Queen, unpublished data), was partially cleaved with Eco RI. The Eco RI site 5' to the  $V_H D_{JH}$  gene segment was converted to a Sal I site by blunting the 5' ends and by addition of Sal I linkers. A pBR322 derivative plasmid, lacking the "poison" sequences between nucleotides 1120 and 2490 was obtained by changing the Eco RI

site in pML1 (Lusky and Botchan, 1981) to a Sal I site. Furthermore, the Nru I site of the plasmid at nucleotide position 973 was converted to a Hind III site. Vector DNA was digested with Sal I and Hind III and ligated with the Sal I/Xho I DNA fragment comprising the  $\mu$ 17.2.25 gene and an Xho I/Hind III DNA fragment containing a modified mouse H4 histone gene (R. Groschedl and D. Baltimore, unpublished data). The resulting clone, with the Sal I site of the  $\mu$  gene fused to the Sal I site of the vector, was called  $\mu$ <sub>μ</sub>.

#### DNA injections into mouse eggs

$\mu$ <sub>μ</sub> DNA was linearized with Sal I and the single-stranded ends were made double-stranded with DNA polymerase (Klenow fragment) and dNTPs. Fertilized one-cell eggs were recovered from the oviducts of C57BL/6J female mice that had mated with C57BL/6J males the previous night. Approximately 1 pl of the linearized  $\mu$ <sub>μ</sub> DNA at a concentration of 0.8  $\mu$ g/ml (or 50 molecules/pl) was microinjected into one pronucleus of each egg as previously described (Costantini and Lacy, 1982).

#### Isolation of DNA

For the isolation of tail DNA, approximately one-third of the tail was cut off, the bone was removed and discarded, and the skin was weighed (the final yield of DNA was 2–3  $\mu$ g per mg of tail skin). The skin was then incubated overnight at 55°C in 1.0 ml of 0.1 M EDTA, 0.05 M Tris-HCl (pH 8), 0.5% sodium dodecyl sulfate, 500  $\mu$ g/ml proteinase K, on a rocking platform; no physical disruption was necessary. The resulting homogenate was centrifuged to remove undigested hair, extracted once with phenol and once with phenol/chloroform. The DNA was precipitated by addition of sodium acetate pH 6.0 to 0.3 M and one volume of ethanol, at room temperature. The DNA pellet was washed once with 70% ethanol, dried and resuspended in 10 mM Tris-HCl (pH 8), 1 mM EDTA. Restriction digestions and DNA/DNA hybridization analyses were performed as previously described (Costantini and Lacy, 1982) except that RNAase A (5  $\mu$ g/ml) was included in the restriction digests to digest residual RNA contaminating the tail DNA samples.

#### Isolation and Purification of RNA

Total RNA was prepared from the various cells and tissues by the guanidium isothiocyanate extraction procedure (Chirgwin et al., 1979) and purified on CsCl gradients (Glisin et al., 1974).

#### Size Analysis of $\mu$ <sub>μ</sub>17.2.25 Specific RNA

Ten micrograms of total RNA was treated with 6% formaldehyde and fractionated by electrophoresis through a 1% agarose gel in 6% formaldehyde, 20 mM phosphate buffer (pH 7.0) (Lehrach et al., 1977) at 100 V for 5 hr. After staining the gel with ethidium bromide and destaining, the RNA was transferred to nitrocellulose paper and hybridized with a <sup>32</sup>P nick-translated, cloned  $\mu$ <sub>μ</sub>17.2.25 specific Pst I DNA fragment as described by Alwine et al. (1977).

#### Perfusion of Liver

Liver was perfused as described (Leffert et al., 1979). The tissue was disaggregated by collagenase treatment and hepatocytes were enriched by centrifugation.

#### Preparation of Cell Populations

Primary fibroblast cells were obtained from muscular fascia. Fibroblasts were grown in DME supplemented with 15% fetal calf serum. Macrophages were prepared from 10<sup>7</sup> peritoneal cells of one thioglycolate-stimulated (Gallilly and Feldman, 1967) transgenic mouse. Cells were collected, washed, and transferred onto a 10 cm tissue culture plate containing RPMI with 10% fetal calf serum and 50  $\mu$ M 2-Mercaptoethanol. After 5 hr of incubation nonadherent cells were removed. One micrometer latex particles were added to a portion of the adherent cells to identify the cells as macrophages by uptake of the latex particles. B cells and T cells were purified from spleen and mesenteric lymph nodes by binding of the cells to plastic dishes coated with F(ab')<sub>2</sub> anti-Ig and F(ab')<sub>2</sub> anti-Lyt 2, respectively, as described (Mage et al., 1977; Wysocki and Sato, 1978; N. Landau, unpublished results).

#### S1 Nuclease Analysis

From 2 to 20  $\mu$ g total RNA was hybridized in 10  $\mu$ l hybridization buffer (Hentschel et al., 1980) with <sup>32</sup>P 5'-end-labeled DNA fragments. The

hybridization temperatures were 38°C for the  $\mu$  DNA probe and 50°C for the  $\kappa$  and beta-2 microglobulin DNA probes. The hybrids were digested with 50 U of S1 nuclease (PL-Biochemicals) at 37°C for 1 hr. The protected DNA fragments were separated by electrophoresis through 8% polyacrylamide-urea gels.

#### Immunoglobulin Radioimmunoassays

NP binding activity assays and immunoglobulin isotype competitive binding assays were conducted on plastic microtiter wells according to White-Scharf and Imanishi-Kari (1982). An NP derivative, NIP (4-hydroxy-5-iodo-3-nitrophenyl)acetyl, coupled to bovine serum albumin (BSA), was provided by Dr. Thereza Imanishi-Kari. In direct binding experiments, wells were coated with 5  $\mu$ g/ml NIP-BSA after which free protein binding sites were blocked with 1% BSA. Serum dilutions were incubated in the wells and subsequently washed with 150 mM NaCl. Wells were then incubated with <sup>125</sup>I-antibody radiolabeled by the chloramine-T method (Greenwood et al., 1963), and <sup>125</sup>I dpm counted after extensive 150 mM NaCl washes. Polyclonal rabbit anti-mouse  $\lambda$ <sub>1</sub>, rat anti-mouse  $\mu$ , or monoclonal antibodies LS136 (anti-mouse  $\lambda$ <sub>1</sub>) or 187.1 (anti-mouse  $\kappa$ ) were iodinated. Serum concentrations of NP binding antibody were estimated by a comparison with purified anti-NP antibody 124/57 ( $\mu$ ,  $\lambda$ <sub>1</sub>) on 18.85 ( $\lambda$ <sub>2B</sub>,  $\kappa$ ). Similarly, wells coated with 10  $\mu$ g/ml anti-BALB/c 17.2.25 immunoglobulin were treated with serum dilutions, and probed with <sup>125</sup>I rat anti-mouse  $\mu$  for NP $\mu$  specific binding. For the isotype competition experiments, wells were coated with 10  $\mu$ g/ml polyclonal rat anti-mouse  $\mu$ , anti-mouse  $\gamma$ <sub>1</sub>, anti-mouse  $\gamma$ <sub>2A</sub>, or anti-mouse  $\gamma$ <sub>2B</sub> antibodies, and free protein binding sites blocked with 1% BSA. Then serial serum dilutions were incubated with an equal volume of <sup>125</sup>I-labeled specific mouse immunoglobulin: P1.37.9 (IgM), B6.63 (IgG<sub>1</sub>), P8.44.18 (IgG<sub>2A</sub>), B6-1E (IgG<sub>2B</sub>), or a mixture of the IgG antibodies on the coated wells. Wells were then washed extensively with 150 mM NaCl before scintillation counting.

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# Immunoglobulin gene transcription ceases upon deletion of a distant enhancer

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The tissue-specific E $\mu$  enhancer within the immunoglobulin heavy chain (IgH) locus has recently been shown to be essential for efficient V region gene assembly in early B lineage cells. However, we and others have shown that late stage, Ig-secreting cells can produce IgH in the absence of E $\mu$ . In the present study we have explored the notion that another enhancer found in the far 3' region of the IgH locus (3' $\alpha$ E) takes on an important regulatory role in cells that have reached this terminal stage in B cell development. The technique of homologous recombination was used to disrupt the 3' $\alpha$ E region in an E $\mu$ -deficient, Ig $\gamma$ 2a-secreting cell line. Loss of 3' $\alpha$ E completely abolished Ig heavy chain gene expression, demonstrating that transcription of this gene was dependent upon sequences that reside over 70 kb downstream. The ability of these sequences to function efficiently in the absence of E $\mu$  may also provide an explanation for deregulated *c-myc* expression in many Ig-secreting tumors.

**Keywords:** 3' $\alpha$ E/E $\mu$ /homologous recombination/*c-myc*/myeloma

## Introduction

Immunoglobulin heavy and light chain genes are unlike most other tissue-restricted genes in that they must be assembled from dispersed gene segments. The assembly of these segments (V and J for Ig light chain, V, D and J for Ig heavy chain) is accomplished through a series of DNA recombination events. V-D-J joining in the immunoglobulin heavy chain (IgH) locus results in the formation of a  $\mu$  heavy chain transcription unit. After Ig light chain gene assembly has also taken place, the developing B lymphocyte begins to display the characteristic IgM molecule on its cell surface, where it serves as an antigen receptor. Antigen- and T lymphocyte-driven events can induce an IgM-positive cell to switch to expression of another class of heavy chain, but with the same antigen specificity. This switch is accomplished through a second type of DNA recombination event, the 'Ig heavy chain class switch' (reviewed in Max, 1993).

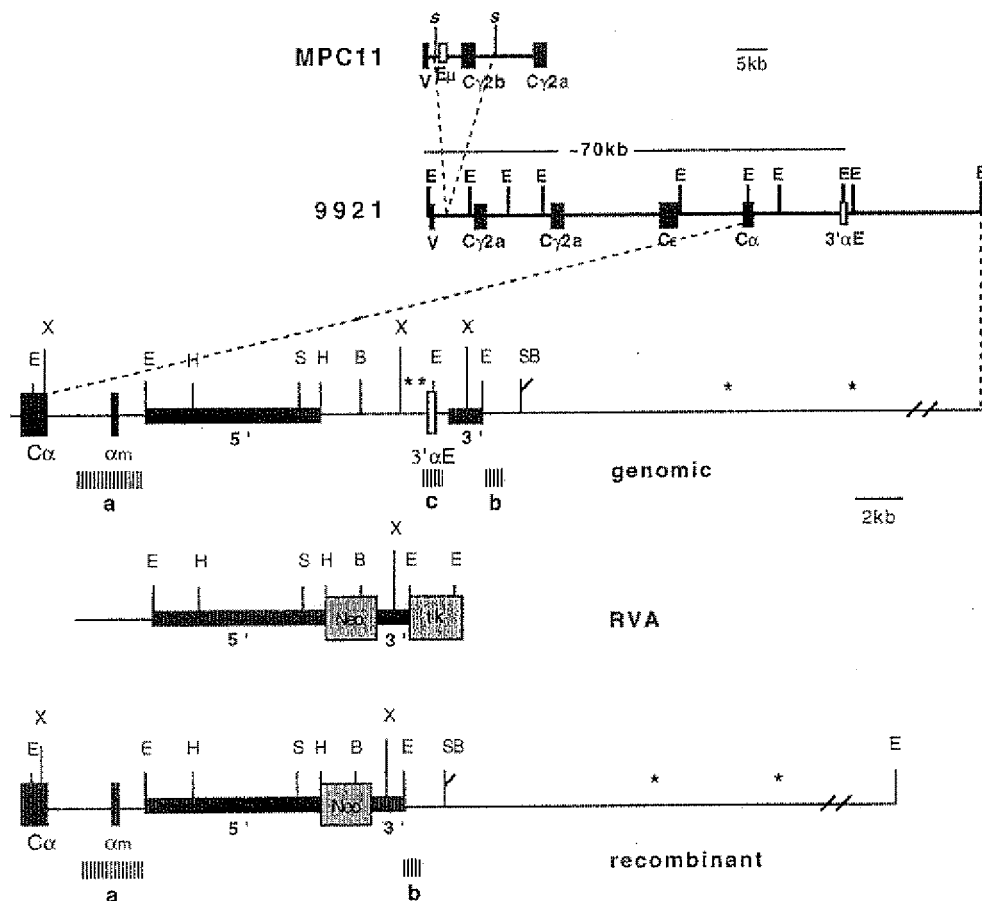
Within the non-coding sequences separating the V<sub>H</sub> and C<sub>H</sub> regions of an assembled  $\mu$  heavy chain gene lies a transcriptional enhancer, E $\mu$ . E $\mu$  was one of the first non-

viral transcriptional enhancers to be described and was the first example of a tissue-specific enhancer (Banerji *et al.*, 1983; Gillies *et al.*, 1983; Neuberger, 1983). While initially identified by virtue of its effects on IgH gene transcription, E $\mu$  has since been shown to enhance the V-D-J recombination process as well (Chen *et al.*, 1993; Serwe and Sablitzky, 1993). This, together with the early discovery that the IgH genes of Ig-secreting cells (a late stage in B lymphocyte development) could function without E $\mu$  (Klein *et al.*, 1984; Wabl and Burrows, 1984; Aguilera *et al.*, 1985; Eckhardt and Birshtein, 1985; Zaller and Eckhardt, 1985), led to the notion that E $\mu$  might serve an important function in early stage B cells, but a less critical one in later stage cells.

One of the first Ig-secreting cell lines shown to secrete Ig in the absence of E $\mu$  was the murine myeloma 9921 (Eckhardt and Birshtein, 1985; Zaller and Eckhardt, 1985). 9921 produces copious amounts of IgG2a. E $\mu$  was lost from the expressed  $\gamma$ 2a gene of 9921 as the result of a heavy chain class switch (see Figure 1). Additional myeloma lines that lacked E $\mu$  were discovered in a number of laboratories, demonstrating that E $\mu$ -independent gene expression was not the peculiar attribute of a single cell line (Klein *et al.*, 1984; Wabl and Burrows, 1984; Aguilera *et al.*, 1985; Klein *et al.*, 1985). While loss of E $\mu$  is not a requisite step in the differentiation of B lymphocytes (the heavy chain class switch usually takes place without loss of E $\mu$ ), the demonstration that IgH genes could function without this enhancer suggested that transcriptional control of these genes was more complex than initially appreciated. As a result, several laboratories began a search for additional enhancers within the IgH locus.

Coinciding with the discovery of E $\mu$ -independent IgH gene expression were descriptions of chromosomal translocations between the IgH locus and the oncogene *c-myc*. Such chromosome translocations are characteristic of both Burkitt's lymphoma (humans) and mouse myeloma (reviewed in Klein and Klein, 1985) and generally lead to deregulated expression of *c-myc*. Furthermore, cell fusion studies showed that the translocated *c-myc* was being expressed in a B cell-specific manner (Greenberg *et al.*, 1989). It was suggested by several groups that juxtaposition of *c-myc* with a B cell-specific control element(s) within the IgH locus was the explanation for abnormal *c-myc* expression in these tumor lines. Although E $\mu$  was initially considered a good candidate, it was subsequently noted that in many mouse myelomas the translocated *c-myc* and E $\mu$  lay on opposite translocation products (reviewed in Cory, 1986). Again, there was an impetus to search for additional IgH locus enhancers.

A few years ago a second DNA segment with transcriptional enhancer activity was identified at the 3'-end of the murine IgH locus (Dariavach *et al.*, 1991; Lieberman *et al.*, 1991). This enhancer region, 3' $\alpha$ E, lies ~200 kb 3' of the

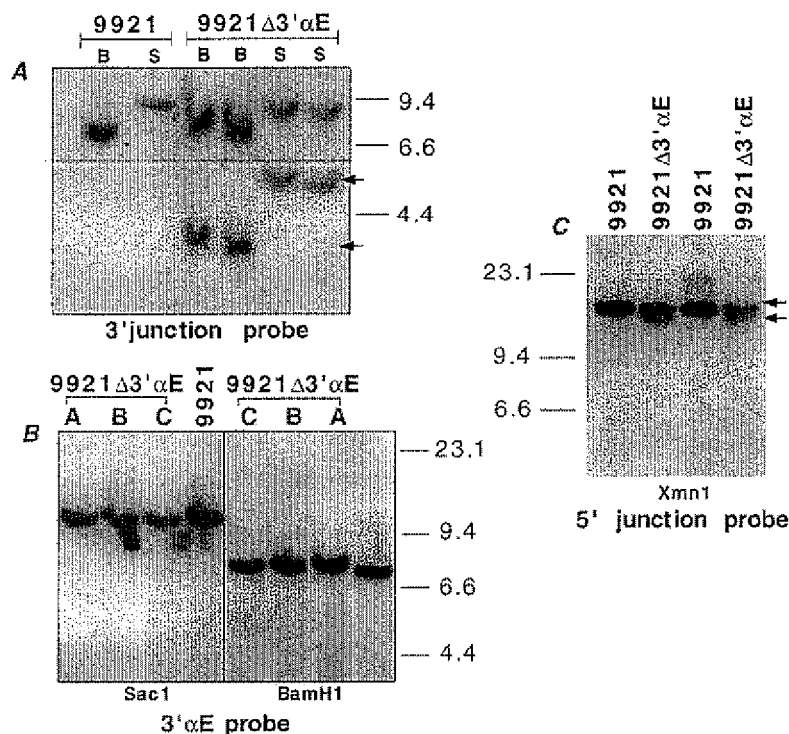


**Fig. 1.** Targeted deletion of the 3'αE region in 9921. MPC11, a γ2b-secreting cell line, gave rise to 9921 through an Ig heavy chain class switch. Relevant regions of the IgH loci in these two cell lines are shown (MPC11 and 9921 maps respectively). The break points of the class switch recombination event are indicated (S). The position of the intronic IgH enhancer Eμ is indicated by an open box, as is the 3' region enhancer (3'αE). The V<sub>H</sub> region expressed in MPC11 and 9921 is shown as a filled box (V); constant region coding sequences are also shown as filled boxes (Cγ2b, Cγ2a, Cα). EcoRI restriction sites are indicated (E) on the 9921 locus map. The distance between 3'αE and the expressed V<sub>H</sub> is >70 kb. Below the MPC11 and 9921 locus maps is a detail of the region 3' of Cα in 9921 (genomic), a map of the replacement targeting vector (RVA) and the predicted structure of the genomic locus after homologous recombination with RVA (recombinant). Thick lines designated 5' and 3' refer to regions of homology between RVA and genomic DNA. The Cα membrane exon is included in the three maps (αm) and 3'αE is designated as an open box. Specific restriction sites which flank 3'αE are indicated and include BamHI (B), EcoRI (E), SacI (S), XmnI (X) and HindIII (H). Except for EcoRI, the map does not include all of the restriction sites for these enzymes. Asterisks indicate the positions of four DNase I hypersensitive sites identified by others (Giannini *et al.*, 1993; Madisen and Groudine, 1994). *pgk-neo* sequences and the MCI-tk gene are shown as shaded boxes (Neo and tk respectively). The HSV-tk gene was included, but not used, in the RVA vector as a means for selecting against cells that had randomly integrated this vector (see Materials and methods). The probes used for Southern blot analysis are represented by vertically striped bars underneath the genomic configurations and are given letter designations (a, b and c).

J<sub>H</sub> gene segments where V<sub>H</sub> gene assembly takes place. As measured in transient transfection assays, 3'αE was found to be less powerful than Eμ in some Ig-secreting cell lines, but equivalent to Eμ in others (Dariavach *et al.*, 1991; Lieberson *et al.*, 1991; Singh and Birshtein, 1993). 3'αE was, at best, a weak transcriptional enhancer in pre-B and surface Ig<sup>+</sup> cell lines (Dariavach *et al.*, 1991; Singh and Birshtein, 1993; Madisen and Groudine, 1994) and a recent study of transgenic mice carrying a 3'αE-controlled reporter gene supports the notion that this element begins to function only after B cells are activated (Arulampalam *et al.*, 1994). Interestingly, analyses of mice in which B lineage lymphocytes lack 3'αE have suggested that 3'αE plays a role in regulating the Ig heavy chain class switch (Cogne *et al.*, 1994). The emerging impression is that 3'αE and Eμ provide essential functions at separate times during B lymphocyte development. Eμ is required for V-D-J joining and for early IgH gene expression; 3'αE

becomes active late in B cell development and participates in the Ig heavy chain class switch.

In the present study we have induced deletion of 3'αE in a cell line (9921) that already lacks Eμ. We undertook these experiments to determine whether 3'αE was responsible for sustaining IgH gene expression in Ig-secreting cells that lacked Eμ. As will be discussed below, 3'αE is not the only enhancer that has been identified 3' of the IgH locus (Giannini *et al.*, 1993; Matthias and Baltimore, 1993; Madisen and Groudine, 1994). However, it was the first to be identified and, therefore, the first candidate considered in our experiments. Deletion of this element *in situ* allowed us to assess 3'αE function within its normal chromosomal context. 3'αE lies 70 kb downstream of the 9921 γ2a gene (Lieberson *et al.*, 1991). The same spacing between enhancer and promoter would be difficult to achieve in conventional reporter gene constructs. Moreover, it is not presently understood whether the nature of



**Fig. 2.** Southern analyses demonstrating targeted deletion of the 3'αE region in 9921. Genomic DNA was isolated from the parental line (9921) and from a G418<sup>r</sup> derivative line (9921Δ3'αE), digested with (A and B) *Bam*HI (B) or *Sac*I (S) or (C) *Xmn*I and size-fractionated on agarose gels. Nylon blots of the DNA were then hybridized to a 3' junction probe (A), 3'αE sequences (B) or to a 5' junction probe (C). The 5' and 3' junction probes are designated probes a and b respectively in the maps of Figure 1. The 3'αE probe is designated c in Figure 1. In (A) the new fragments generated upon homologous recombination of RVA with genomic sequences are indicated by arrows. In (B) DNAs from three subclones of the 9921Δ3'αE line are given letter designations (A, B and C). The two arrows in (C) mark the closely migrating *Xmn*I fragments derived from IgH chromosomes that retain the 3'αE region (upper fragment) and the IgH chromosome that has undergone replacement of the 3'αE region with *neo* sequences (lower fragment) respectively. Molecular size markers indicated (23.1, 9.4, 6.6 and 4.4 kb) are *Hind*III fragments of bacteriophage λ.

the DNA separating an assembled IgH gene and a distal enhancer, such as 3'αE, might affect their interactions with one another (e.g. the presence of matrix attachment sites; Cockerill, 1990).

Our studies show that 3'αE is required for γ2a gene transcription in the 9921 cell line. Targeted deletion of a 5.1 kb region that included 3'αE gave rise to a cell line that produced normal levels of Ig κ light chain, but which had ceased production of γ2a heavy chain. This DNA region, therefore, strongly regulates the activity of a promoter over 70 kb away. As discussed below, the finding that 3'αE behaves as a strong transcriptional enhancer *in situ* and in the absence of Eμ is also of relevance to the deregulated expression of translocated *c-myc* genes in tumors of the B cell lineage.

## Results

### Isolation of a 9921 subclone that has undergone deletion of 3'αE sequences

In order to induce deletion of 3'αE from the active IgH locus of 9921, we constructed the targeting vector replacement vector A (RVA; Figure 1). This construct was designed so that recombination between it and the targeted chromosome would result in replacement of 5.1 kb of genomic DNA with 1.9 kb of exogenous DNA encompassing a selectable marker gene (neomycin resistance gene, *neo*<sup>r</sup>). The genomic DNA to be deleted included

3'αE and two overlapping DNase I hypersensitive sites identified by others (HS1 and HS2; Giannini *et al.*, 1993; Madisen and Groudine, 1994).

The regions of homology between the RVA targeting vector and genomic sequences consisted of 7 kb of 5' flanking sequence and 1.35 kb of 3' flanking sequence (see Figure 1). The *neo*<sup>r</sup> gene lying between these flanking sequences was under the control of the phosphoglycerate kinase gene (*pgk*-1) promoter region. The RVA vector was linearized and introduced into 9921 cells by electroporation. A genomic DNA screen was used to identify geneticin-resistant (G418<sup>r</sup>) transformants that had undergone the desired homologous recombination event. As diagrammed in Figure 1, homologous recombination between RVA and genomic DNA should result in novel *Bam*HI (3.7 kb) and *Sac*I (5.9 kb) fragments that would hybridize with a '3' junction' probe (probe b in Figure 1). DNA from one of 450 screened clones generated the expected *Bam*HI fragment and was further analyzed with the *Sac*I enzyme (Figure 2A). A *Sac*I fragment of the expected size (5.9 kb) was also detected in this clone, so that it was provisionally designated 9921Δ3'αE. As would be predicted, neither the novel *Sac*I fragment nor the novel *Bam*HI fragment hybridized to a 3'αE probe (Figure 2B).

Southern analyses of 9921Δ3'αE revealed not only the novel *Bam*HI and *Sac*I fragments associated with the homologous recombination event, but also the *Bam*HI and *Sac*I fragments characteristic of non-transfected 9921 cells

(Figure 2A). This was an expected finding because of the chromosomal constitution of the 9921 cell line. One copy of the IgH chromosome (chromosome 12) in 9921 carries the functionally rearranged and expressed  $\gamma 2a$  heavy chain gene. The other copy of chromosome 12, harboring the 'excluded' (non-expressed) IgH locus, has undergone reciprocal translocation with *c-myc* on chromosome 15 (see maps, Figure 4A; Stanton *et al.*, 1984). Subsequent to this translocation event, the reciprocal translocation products have undergone reduplication and, in previous studies, we have estimated that both are present at two or three copies per cell (Stanton *et al.*, 1984). One of the translocation products (designated M11myc3' in Stanton *et al.*, 1984) retains IgH locus sequences homologous to the RVA vector ('Translocated' chromosome in Figure 4A). As a result, RVA could recombine with either this translocated chromosome or with the copy of chromosome 12 carrying the functional  $\gamma 2a$  gene ('Nontranslocated' in Figure 4A). Regardless of which chromosome underwent recombination with RVA, two or more additional chromosomes would retain the 3'  $\alpha E$  region in its original configuration. As a result, the pattern of hybridization to the 3' junction probe should include both the original fragments detected in 9921 and the novel fragments resulting from RVA/genomic DNA recombination. Certainly, only when RVA recombined with the non-translocated chromosome carrying the functional  $\gamma 2a$  gene would it be expected to affect IgH gene expression. This issue is considered in detail below. However, we first extended our analysis of the recombination event in 9921 $\Delta 3'\alpha E$  to confirm that the expected replacement of sequences had taken place.

Having determined that the 3' flanking sequences of RVA had homologously recombined with IgH chromosomal sequences, we next looked for evidence that a second cross-over event had taken place within the RVA 5' flanking sequences. As diagrammed in Figure 1, the expected recombination event would generate a novel *XmnI* fragment that could be detected with a '5' junction' probe (probe a, Figure 1). The new *XmnI* fragment should be slightly smaller than that which would be detected with the 'a' probe before recombination (compare 'genomic' and 'recombinant' maps, Figure 1). When 9921 $\Delta 3'\alpha E$  DNA was digested with *XmnI*, the expected pattern of two closely migrating fragments was seen, confirming that this cell line carried an IgH chromosome that had undergone the desired homologous recombination event (Figure 2C).

The original 9921 $\Delta 3'\alpha E$  isolate was subcloned by limiting dilution to ensure that it was a homogeneous population of cells. Genomic DNA analyses of each of the subclones showed the same patterns as those of the original cell isolate. Interestingly, hybridizations with a *neo*<sup>r</sup> probe revealed that additional copies of the RVA vector had been integrated at random sites within the 9921 $\Delta 3'\alpha E$  genome (data not shown).

#### **9921 $\Delta 3'\alpha E$ and subclones no longer produce $\gamma 2a$ mRNA or protein**

To determine whether the loss of 3'  $\alpha E$  sequences in 9921 $\Delta 3'\alpha E$  had affected  $\gamma 2a$  expression, we prepared cytoplasmic lysates and culture supernatants from five 9921 $\Delta 3'\alpha E$  subclones. These were analyzed by enzyme-linked immunoassay (ELISA) for  $\gamma 2a$  protein (Figure 3A).

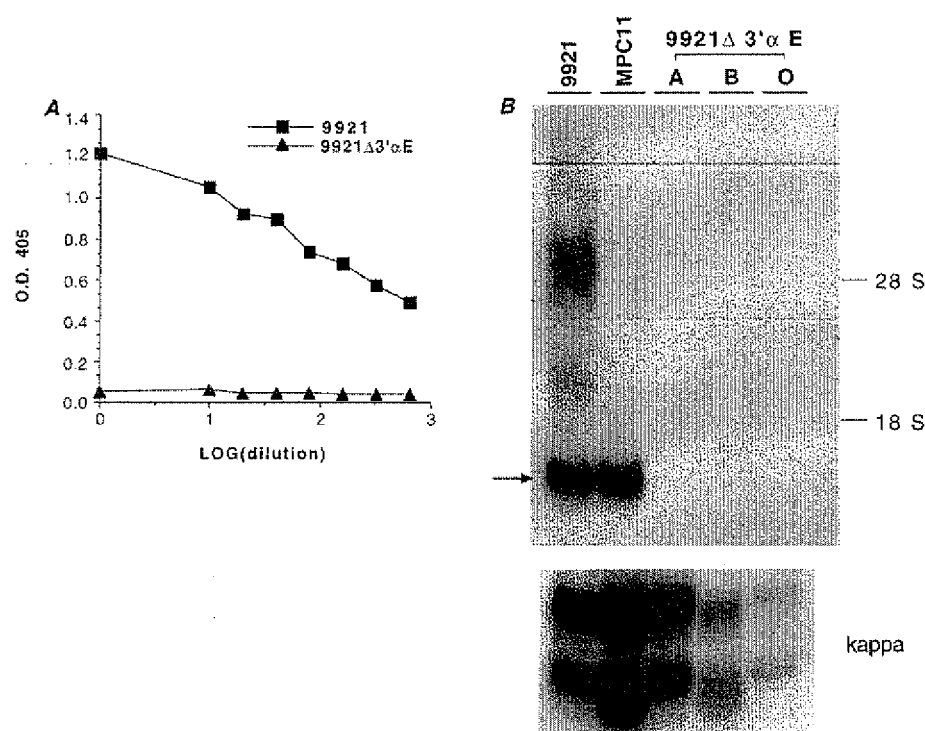
While lysates and supernatants from the parental 9921 line and from the MPC11 myeloma contained abundant  $\gamma 2a$  and  $\gamma 2b$  heavy chain protein respectively, no Ig heavy chain was detected in any of the 9921 $\Delta 3'\alpha E$  subclones (data for 9921 and a representative 9921 $\Delta 3'\alpha E$  subclone, Figure 3A). Furthermore, there was no  $\gamma 2a$  mRNA in RNA samples prepared from three representative 9921 $\Delta 3'\alpha E$  subclones (Northern blot analyses, Figure 3B).  $\gamma 2a$  expression was specifically affected in these cell lines. Quantitative ELISAs demonstrated that all clones continued to produce  $\kappa$  light chain protein at the same levels as 9921 (data not shown) and  $\kappa$  mRNA was readily detectable by Northern analyses (Figure 3B).

It has been shown previously that myelomas give rise to Ig non-producing cell lines at a relatively high rate ( $1/10^3$ /cell/generation; Coffino and Scharff, 1977). While this rate is well above that for other loci in these or other cell types (generally  $1/10^6$ /cell/generation), it is low enough to suggest that we would not recover cells that had spontaneously lost Ig expression in a random screen of 450 myeloma subclones. Consistent with this prediction, we found that in a screen of 325 random transformants of the 9921 cell line, none had ceased to express Ig. Each of the transformants included in this screen had been transfected with plasmid DNA and isolated by growth in selective growth medium. However, none of them had undergone homologous recombination with the RVA vector (data not shown).

In almost all cases in which spontaneous Ig non-producing clones have arisen in cultures of myeloma cell lines (18 in a study of 19 spontaneous myeloma variants), the loss of IgH expression has resulted from detectable alterations in DNA: either loss of the relevant IgH chromosome or DNA rearrangements within or around the Ig heavy chain transcription unit (Yu and Eckhardt, 1986). We analyzed 9921 $\Delta 3'\alpha E$  DNA, therefore, for evidence of either of these events. 9921 $\Delta 3'\alpha E$  and parental 9921 DNA were both digested with *Bam*HI and *Sac*I and hybridized with a DNA probe (pJ11) that detects sequences within the  $\gamma 2a$  transcription unit of 9921 (see map, Figure 4A). 9921 and the 9921 $\Delta 3'\alpha E$  subclones were indistinguishable in these analyses, demonstrating that the 9921 $\Delta 3'\alpha E$  subclones retained the  $\gamma 2a$  gene actively transcribed in 9921 (Figure 4B). The lack of transcription at this locus in 9921 $\Delta 3'\alpha E$  and subclones, therefore, is not likely to be due to spontaneous mutational events, but rather results from the homologous recombination event that has deleted 3'  $\alpha E$ -region sequences ~70 kb downstream.

#### **RVA-induced deletion of 3' $\alpha E$ has occurred in cis to the $\gamma 2a$ gene expressed in 9921**

As noted earlier, the expressed  $\gamma 2a$  gene of 9921 lies on an intact copy of chromosome 12 with 3'  $\alpha E$  lying ~70 kb downstream (with respect to IgH gene transcription). The homologous IgH chromosome has undergone reciprocal translocation with chromosome 15 such that one of the translocation products (M11myc3') retains 3'  $\alpha E$  and flanking sequences. The translocation has resulted from a break within the tandemly repetitive sequences 5' of  $\gamma 2a$  (S $\gamma 2a$ ) on the IgH chromosome and a break within exon 1 of *c-myc* on chromosome 15 (Stanton *et al.*, 1984). The M11myc3' translocation product carries 3'  $\alpha E$  in cis to the disrupted *c-myc* gene (~50 kb upstream with respect to



**Fig. 3.**  $\gamma 2a$  heavy chain expression has ceased in 9921 $\Delta 3'\alpha E$ . (A) Quantitative ELISA comparing  $\gamma 2a$  protein levels in 9921 and 9921 $\Delta 3'\alpha E$ . Titration curves of cytoplasmic lysates from 9921 and from a representative 9921 $\Delta 3'\alpha E$  subclone are shown. Culture supernatants and cytoplasmic lysates from four additional 9921 $\Delta 3'\alpha E$  subclones were also tested by quantitative ELISA for the presence of  $\gamma 2a$  protein and similar results were obtained.  $\gamma 2a$  heavy chain was detected with an alkaline phosphatase-conjugated rabbit antibody (Materials and methods). The reaction of this reagent with *p*-nitrophenol phosphate yields a colored product with an absorbance peak at 405 nm. (B) Northern blot analysis of total RNA isolated from 9921, the myeloma cell line MPC11 ( $\gamma 2b$ ,  $\kappa$  producer) and three representative 9921 $\Delta 3'\alpha E$  subcloned lines (designated A, B and O). Aliquots of 20  $\mu$ g total cellular RNA were included in each sample. Positions of ribosomal RNAs (28S and 18S) are indicated. The probe used in the upper blot was a  $\gamma 2a$  cDNA probe (Zaller and Eckhardt, 1985) which also cross-hybridizes to  $\gamma 2b$  transcripts. An arrow points to the  $\gamma 2a$  and  $\gamma 2b$  mRNA present in 9921 and MPC11 respectively. Subsequent hybridization of the same Northern blot with a probe specific for  $\kappa$  light chain mRNA, C $\kappa$  (Radomska *et al.*, 1994), revealed the two  $\kappa$  light chain mRNAs characteristic of the MPC11 lineage (including 9921). The faster migrating band is a truncated  $\kappa$  mRNA from the non-productive Ig  $\kappa$  allele; the more slowly migrating mRNA encodes functional  $\kappa$  light chain (Schibler *et al.*, 1978).

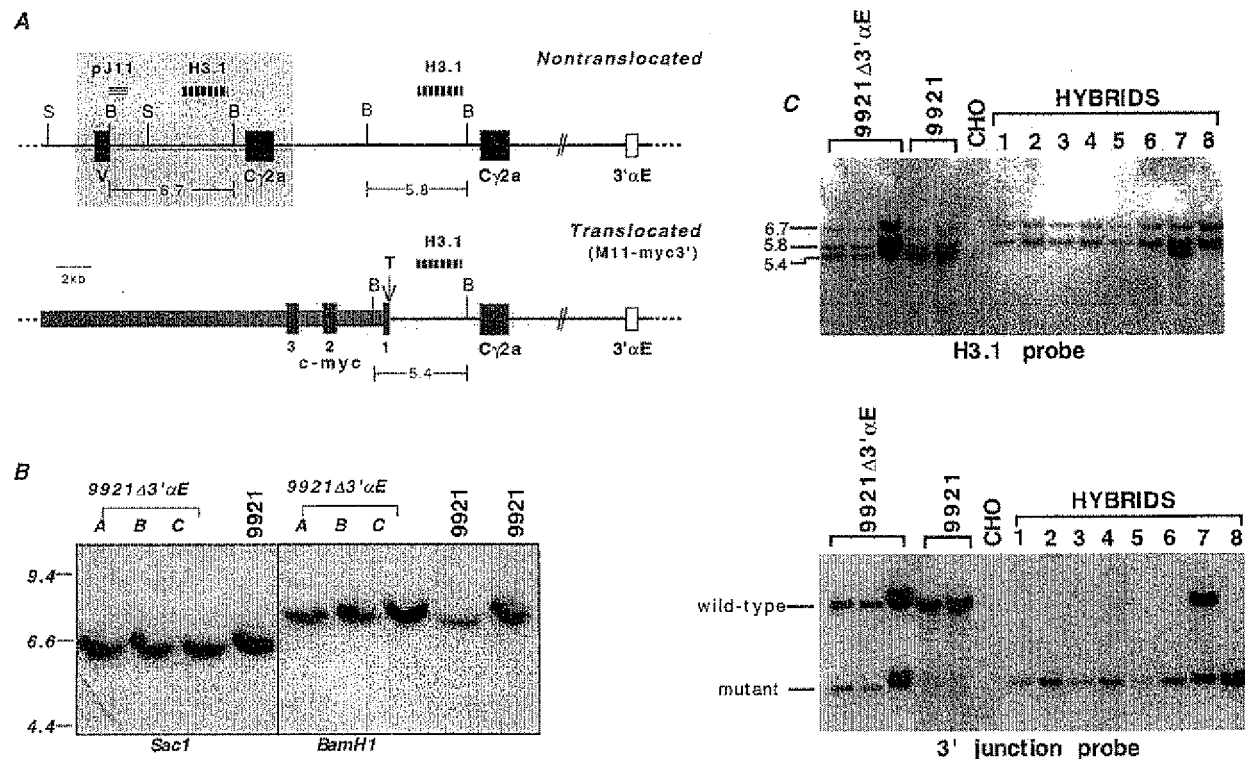
*c-myc* transcription; see map, Figure 4A). Our Southern blot data for 9921 $\Delta 3'\alpha E$  indicated that one copy of 3'αE and its flanking sequences had been replaced by *neo<sup>r</sup>* but that other copies of 3'αE remained intact in this cell line. Although the loss of  $\gamma 2a$  expression in 9921 $\Delta 3'\alpha E$  and its subclones strongly implied that the deletion had occurred *in cis* to the  $\gamma 2a$  heavy chain gene on the non-translocated IgH chromosome, we undertook experiments to physically map the deletion to this or the translocated chromosome, M11myc3'.

M11myc3' and the  $\gamma 2a$  gene-containing IgH chromosomes are nearly identical throughout at least a 50 kb region surrounding 3'αE. This made it difficult to link *neo<sup>r</sup>* sequences to one or the other chromosome by conventional genomic Southern analysis. Instead, we elected to use the method of somatic cell fusion as a means to physically separate the two chromosomes so that we could unambiguously map the homologously recombined copy of RVA to one or the other of these chromosomes. 9921 $\Delta 3'\alpha E$  cells (G418<sup>r</sup>) were fused to ouabain-resistant, Chinese hamster ovary (CHO, Ouabain<sup>r</sup>) cells with the expectation that the resulting hybrids would have lost murine chromosomes during the process of cell fusion (Ruddle, 1972). An electrofusion method developed in our laboratory (Radomska *et al.*, 1994; Radomska and Eckhardt, 1996) was used for these experiments and

hybrids were isolated after growth in medium containing ouabain and G418 (see Materials and methods). The use of G418 allowed us to select for hybrids that retained the chromosome in which 3'αE had been replaced by *neo<sup>r</sup>*.

DNA samples from individual hybrid lines were subjected to Southern blot analysis using the *Bam*HI restriction enzyme and the H3.1 probe indicated in Figure 4A. The H3.1 probe hybridizes to two *Bam*HI fragments (6.7 and 5.8 kb) that map within the non-translocated ( $\gamma 2a$  gene-containing) IgH chromosome and to a 5.4 kb *Bam*HI fragment from M11myc3'. The latter *Bam*HI fragment spans the translocation break point within the *c-myc* gene. It is possible to determine which hybrid lines retain which of these two IgH chromosomes (translocated or non-translocated) by determining which *Bam*HI fragments remain (i.e. all three, 6.7 and 5.8 kb only or 5.4 kb only).

Representative data from these analyses are shown in Figure 4C. All of the tested CHO×9921 $\Delta 3'\alpha E$  hybrids (14/14) retained the 6.7 and 5.8 kb *Bam*HI fragments, demonstrating that all retained the intact copy of chromosome 12 carrying the expressed  $\gamma 2a$  gene. However, most of the hybrids (12/14) had lost all copies of M11myc3', as indicated by the absence of the 5.4 kb *Bam*HI fragment in these DNA samples. Preferential retention of the chromosome carrying the  $\gamma 2a$  gene in the hybrid lines indirectly suggested that RVA (with *neo<sup>r</sup>*) had integrated



**Fig. 4.** Deletion of the 3'αE region has occurred *in cis* to the γ2a IgH transcription unit. (A) Two schematics of 9921 chromosomes are shown. One is of the IgH chromosome that carries the functional γ2a gene of 9921 ('Nontranslocated'). The γ2a transcription unit on this chromosome is highlighted. The allelic IgH chromosome in 9921 has undergone a reciprocal translocation with chromosome 15. The translocation break points map within *c-myc* (on chromosome 15) and upstream of *Cγ2a* (on chromosome 12) (Stanton *et al.*, 1984). The second schematic is of one of the products of this reciprocal translocation ['Translocated' (M11-myc3')]; Stanton *et al.*, 1984]. T marks the site of recombination between chromosomes 15 and 12. The thick shaded line signifies sequences derived from chromosome 15. *V<sub>H</sub>*, *Cγ2a* and *c-myc* exons are shown as black boxes. The *Bam*HI (B) and *Sac*I (S) restriction enzyme sites germane to the Southern analysis shown in (B) and (C) are indicated. Sequence homology to the pJ11 and H3.1 DNA probes is indicated above each map. The sizes of *Bam*HI fragments detected with these two probes are indicated below the maps (in kb). It should be noted that a second copy of *Cγ2a* lies 3' of the γ2a transcription unit in 9921. This is the result of a tandem duplication which originally took place in MPC11, the cell line from which 9921 was derived (Tilley and Birshtein, 1985). Due to this duplication the H3.1 probe detects two distinct *Bam*HI fragments that come from the non-translocated (γ2a-producing) IgH chromosome. (B) Southern analyses of 9921 and 9921Δ3'αE subclones using a DNA probe specific for the γ2a transcription unit. DNAs from the cell lines were digested with *Sac*I (left) or *Bam*HI (right) and hybridized to pJ11 (see map in A). Molecular weight markers are as in Figure 2. (C) Southern analyses of CHO×9921Δ3'αE hybrids. *Bam*HI-digested DNA was prepared from 9921Δ3'αE, 9921, CHO and several individual hybrid lines (hybrids 1–8 are shown). Duplicate blots were prepared and one of these hybridized with the H3.1 probe. The *Bam*HI fragments detected with this probe are indicated (5.4, 5.8 and 6.7 kb) and correspond to the fragments diagrammed in (A). A duplicate blot was hybridized with the 3' junction probe (probe b in Figure 1). The two *Bam*HI fragments detected with this probe come from a region 3' of *Cα* and signify either RVA-induced disruption of this region ('mutant' fragment) or no disruption of 3'αE flanking sequences ('wild-type' fragment).

into that chromosome. This was confirmed by additional Southern blot analyses.

Duplicate samples of *Bam*HI-digested genomic DNA were hybridized with the 3' junction probe (probe b in Figure 1) to determine which hybrids carried the mouse chromosome that had homologously recombined with RVA. Since analyses with the H3.1 probe showed that all hybrids retained the non-translocated IgH chromosome (with the assembled γ2a gene), the prediction was that all hybrids would also retain the diagnostic *Bam*HI fragment associated with RVA-induced deletion of 3'αE, if this deletion had occurred *in cis* to the γ2a gene. Consistent with that prediction, all 14 hybrids analyzed retained the novel 3.7 kb *Bam*HI fragment derived from the RVA-targeted allele (Figure 4C; compare with Figure 2A). Notably many of these hybrids lacked M11myc3', proving that RVA had not homologously recombined with this translocated chromosome. Rather, the 6.9 kb *Bam*HI

fragment characteristic of an undisrupted 3'αE region was mapped to the M11myc3' chromosome, since this *Bam*HI fragment was present only in those two hybrids that also retained a copy of M11myc3' (data are shown for only one of these two hybrids, Figure 4C lane 7). Taken together, these data clearly show that RVA-induced deletion of the 3'αE region has occurred *in cis* to the γ2a transcription unit, which has simultaneously ceased to function.

## Discussion

Over 10 years ago we and others described a number of Ig-producing cell lines that expressed Ig heavy chain in the absence of Eμ (Klein *et al.*, 1984; Wabl and Burrows, 1984; Aguilera *et al.*, 1985; Zaller and Eckhardt, 1985). At the time this was the only known transcriptional enhancer within the IgH locus. The discovery that IgH genes could function without Eμ provided the earliest

indication that there were additional elements participating in the control of IgH gene expression.

In recent years several additional enhancers have been mapped to the IgH locus, but their respective roles in promoting and/or regulating endogenous IgH gene expression remain unclear. To explore further the function of one of these enhancers, 3'αE, we have made use of one of the Eμ-independent cell lines, 9921. 9921 expresses γ2a heavy chain from an IgH allele that lacks Eμ. In fact, Eμ is altogether missing from the genome of this cell line (Eckhardt and Birshtein, 1985). 3'αE, on the other hand, is on both the IgH allele that produces γ2a transcripts and on a homologous allele, where it lies 50 kb upstream of a translocated *c-myc* locus. In this study we have shown that when the 3'αE region *in cis* to the γ2a gene is replaced with foreign DNA sequences the γ2a gene ceases to function. This finding strongly supports the notion that this distant 3' region serves an essential function in the transcription of assembled IgH genes.

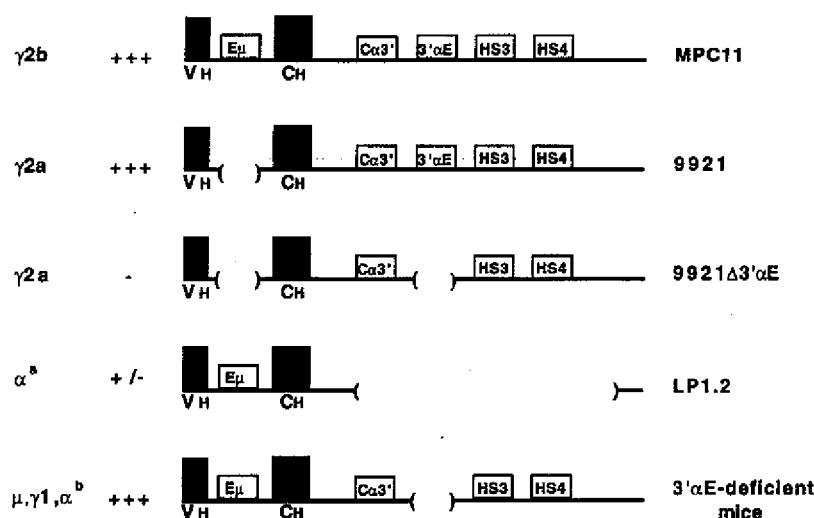
There have been a number of experiments involving mice in recent years which also bear on the question of enhancer function within the IgH locus. Chimeric mice have been produced in which B lymphocytes lacked Eμ on one of the IgH alleles. These mice showed greatly impaired V-D-J joining on the Eμ-deficient IgH chromosome, suggesting both that efficient V region gene assembly requires Eμ and that enhancer regions 3' of the IgH locus cannot supplant Eμ in this process (Chen *et al.*, 1993; Serwe and Sablitzky, 1993). In other recent experiments in mice, 3'αE and surrounding sequences (5.5 kb deletion) were deleted in the lymphoid progenitors of chimeric mice (Cogne *et al.*, 1994). In these mice there was no apparent effect on V-D-J joining, but Ig heavy chain class switching was impaired. The emerging picture is one in which Eμ plays important roles in the activation and regulation of IgH genes in early B cells, while 3' IgH region sequences take on greater importance as B cells mature and/or are activated by antigen.

A limitation to studies involving gene manipulations in mice is that a block in cell development, due to loss of a necessary gene or control region, precludes analysis of the same gene's or control region's function at later developmental stages of the same cell lineage. However, this limitation can be overcome by studies of appropriate cell lines. For example, spontaneous deletion of Eμ within an assembled heavy chain gene of several pre-B cell lines resulted in loss of IgH gene expression, demonstrating that Eμ not only serves to promote efficient V-D-J assembly (as suggested by the mouse studies), but is also required for transcription of IgH genes soon after they are formed (Alt *et al.*, 1982; Wabl and Burrows, 1984). Interestingly, the Eμ-deficient IgH gene of one of these pre-B cell lines could be reactivated under conditions that would also activate 3'αE (Alt *et al.*, 1982; Wabl and Burrows, 1984; Arulampalam *et al.*, 1994). The 3'αE deletion studies in mice suggest that the 3' IgH region, like Eμ, plays an important role in DNA recombination (in this case heavy chain class switch recombination; Cogne *et al.*, 1994). However, the results presented here reveal an additional, important function for 3'αE as a transcriptional enhancer for the assembled IgH genes expressed in Ig-secreting cells.

It is important to point out that in the experiments we

describe here and in the 3'αE deletion in mice (Cogne *et al.*, 1994), a selectable marker was inserted in place of deleted genomic DNA sequences. This was also true for one of the Eμ deletion studies in mice (Chen *et al.*, 1993). In all of these cases it is possible that substitution of the respective enhancers with a marker gene (in our case *neo<sup>r</sup>*) does not constitute a true 'null' mutation. It has been reported that insertion of marker genes within the locus control region (LCR) of the β-like globin locus can, by itself, negatively affect expression of the β-like globin genes (Kim *et al.*, 1992; Fiering *et al.*, 1993). These results were interpreted as evidence that the LCR elements could be redirected to serve a more proximal promoter (that of the reporter gene) when the latter was provided by gene insertion. Certainly, this finding raises the possibility that the effects we have seen in 9921 and that were seen in mice upon 3'αE deletion were due to diversion of control elements that map outside the deletion itself, away from the IgH promoter. This possibility can be directly tested by modification of the RVA replacement vector to permit subsequent deletion of *neo<sup>r</sup>*, and such studies are underway. In any case, there are examples in which insertion of reporter genes within the IgH locus had little or no effect on Ig gene activity (Baker *et al.*, 1988; Chen *et al.*, 1993). The surprising effect of reporter gene insertion in the β-like globin locus LCR may be related to the fact that stage-specific control of the β-like globin genes relies directly upon competitive interactions between promoters and enhancers.

While our studies demonstrate that disruption of the 3'αE region silences the γ2a gene in 9921 cells, it should not be assumed, on the basis of this finding, that 3'αE is sufficient to sustain expression of this gene. In our earlier studies of 3'αE we found it to be a less potent enhancer than Eμ in transient assays in the J558L myeloma cell line (Lieberson *et al.*, 1991). Stable transfection assays in 9921 using a reporter gene under the control of Eμ or of a 4 kb *Xba*I fragment containing 3'αE also suggest that 3'αE is a less efficient transcriptional enhancer (R. Lieberson and L.A. Eckhardt, unpublished results). However, several additional regulatory elements have recently been described in the 3' flanking region of the IgH locus, any one or all of which could work together with 3'αE to achieve high level IgH gene transcription in Ig-secreting cells. A region with relatively weak transcriptional enhancement activity has been identified immediately 3' of Cα membrane sequences (Cα3'E; Matthias and Baltimore, 1993). In addition to the DNase I hypersensitive sites that map within and adjacent to 3'αE (HS1 and HS2), there are two DNase I hypersensitive sites that map 13 (HS3) and 17 kb (HS4) farther downstream (Giannini *et al.*, 1993; Madisen and Groudine, 1994). HS3 was identified in a number of myeloma cell lines, but was absent in DNA preparations from both non-B cells and earlier stage B cells (Madisen and Groudine, 1994). HS4 was identified as a B cell-specific site present in both pre-B and plasmacytoma lines and acts as an enhancer element in transfection assays (Giannini *et al.*, 1993; Madisen and Groudine, 1994; Michaelson *et al.*, 1995). The combination of four DNase I hypersensitive sites (HS1, HS2, HS3 and HS4) has recently been described as an LCR, due to its ability to promote copy number-dependent, insertion site-independent expression of



**Fig. 5.** Schematic of spontaneous and induced deletions of IgH regulatory regions in late stage B cells. The IgH loci in several Ig-producing cells are diagrammed (not to scale). Heavy chain variable (V<sub>H</sub>) and constant (C<sub>H</sub>) region coding sequences are represented as filled boxes. The relative positions of the five control elements discussed in the text (E<sub>μ</sub>, Cα3', 3'αE, HS3 and HS4) are shown as open boxes. The names of the Ig-secreting cell lines are to the right of each map and the classes of heavy chain they produce are indicated to the left. Levels of heavy chain mRNA and protein in the cell lines are represented as +++ (for high level expression), +/- (low expression) and - (no expression). <sup>a</sup>The deletion variant LP1.2 produces α heavy chain at 10% of the level of the original parental cell line (Gregor and Morrison, 1986). <sup>b</sup>3'αE-deficient spleen cells from chimeric mice, when stimulated with LPS and lymphokines, secreted IgM, IgG<sub>1</sub> and IgA at levels comparable with those produced by wild-type spleen cells (Cogne *et al.*, 1994).

reporter genes in myeloma cells (Madisen and Groudine, 1994). If, as suggested by the latter studies, 3'αE is only one component of a larger functioning unit (LCR), then deletion of other single elements might yield the same phenotype we describe for 3'αE (loss of IgH gene expression). This is an issue that can be addressed in future deletion experiments in Ig-secreting cell lines.

While 3'αE is essential for IgH expression when E<sub>μ</sub> is absent, it would appear that the other 3' region elements (e.g. Cα3'E, HS3 and HS4) can function without 3'αE when E<sub>μ</sub> is present. In mice carrying the 3'αE deletion (replacement) but with E<sub>μ</sub> intact, spleen cells expressing some heavy chain isotypes (e.g. IgM, IgG<sub>1</sub> and IgA) were reported to do so at normal levels, suggesting that 3'αE was not required for expression of these genes (see Figure 5). However, it cannot be concluded that E<sub>μ</sub> alone was sustaining IgH expression in these cells. Several years ago a spontaneous deletion of sequences 3' of Cα was described in an IgA-secreting cell line (LP1.2; Gregor and Morrison, 1986). Although E<sub>μ</sub> remained within the α heavy chain gene of this cell line, the deletion of 3' region sequences resulted in a dramatic reduction in IgH expression levels (to 10% of that of the parental line; Gregor and Morrison, 1986). The DNA deletion in LP1.2 involved ~40 kb, began just upstream of the Cα membrane exon and included all of the putative control elements that have been subsequently mapped 3' of Cα (Figure 5). Without any of these 3' region sequences it appeared that E<sub>μ</sub> was insufficient to maintain the high levels of IgH expression characteristic of Ig-secreting cells. Similarly, E<sub>μ</sub> may not act alone to achieve high level expression of IgH genes in the 3'αE deletion mice, but rather it may act in synergy with the remaining 3' region control sequences (Figure 5). In summary, the regulation of IgH gene assembly, rearrangement and transcription in developing and activated B cells appears to require the

action of multiple control elements. Further experiments are required to determine the precise nature of the contributions made by each of these elements to these several processes.

There is reason, separate from their relevance to IgH gene expression, to pursue experiments that allow analysis of 3' IgH region function in the absence of E<sub>μ</sub>. As noted in the Introduction, *c-myc* translocations with the IgH locus in mouse myeloma and human Burkitt's lymphoma frequently juxtapose *c-myc* not with E<sub>μ</sub>, but with the much farther 3' region of the IgH locus (reviewed in Cory, 1986). In this case oncogenic transformation of Ig-expressing cells results, in part, from deregulation of the *c-myc* gene upon placing it under 3' IgH region control. It will be interesting to determine whether deletion of 3'αE and the HS1 and HS2 sites has as dramatic an effect on translocated *c-myc* expression in 9921 as it has on IgH expression.

## Materials and methods

### Cell lines

9921 is a γ2a/κ-producing Ig class switch variant which arose spontaneously from the cell line 971. 971 was, in turn, isolated from mutagenized 45.6.2.4 cells. This lineage is described in Eckhardt and Birshstein (1985). 45.6.24 is a tissue culture-adapted subline of the γ2b/κ-producing BALB/c mouse tumor MPC11 (Laskov and Scharff, 1970). In the present study we refer to this cell line as MPC11. OY21 is a ouabain-resistant variant of the Chinese hamster cell line CHO (a gift of L.Chasin; Urlaub and Chasin, 1980). 9921 was maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Islands, NY) with 15% horse serum (HyClone Laboratories, Logan, UT). OY21 was maintained in RPMI 1640 (Gibco-BRL, Grand Islands, NY) with 10% horse serum. All media contained 100 U/ml penicillin/streptomycin and 2 mM L-glutamine. Cells were maintained at 37°C in an atmosphere of 7% CO<sub>2</sub>.

### Plasmid constructs

RVA was designed to induce a recombination event in which a 1.9 kb neomycin gene (*neo*<sup>r</sup>) would replace 5.1 kb of DNA encompassing 3'αE

(Lieberson *et al.*, 1991) and DNase I hypersensitive sites 1 and 2 (HS1 and HS2; Madisen and Groudine, 1994). RVA was constructed such that the *neo*<sup>r</sup> gene lay between cloned genomic DNA sequences that mapped immediately upstream (5' flanking sequence) and downstream (3' flanking sequence) of the desired genomic DNA deletion. The 5' flanking sequence consisted of a 7 kb genomic DNA fragment isolated from the phage clone pAM2 (a gift of S. Pettersson and M. Neuberger; Dariavach *et al.*, 1991). This fragment was subcloned into the *Eco*RI and *Hind*III sites in the multiple cloning site region of pBS SK<sup>+</sup> (Catalog No. 21120, Stratagene, La Jolla, CA). The resulting plasmid was designated pRV5'. The 3' flanking sequence was a 1.35 kb *Apa*I–*Eco*RI fragment which was also subcloned from the pAM2 phage. This fragment was introduced into the *Hind*III site of the pIC19R/MC1-TK plasmid by blunt-end ligation (pIC19R/MC1-TK was kindly provided by M. Cappechi; Mansour *et al.*, 1988). The resulting plasmid (pRVA-3'tk) carried both the desired 3' flanking region and the negative selection marker, HSV-tk (McKnight, 1980). A 3.5 kb *Xho*I–*Sall* fragment from pRVA-3'tk was then inserted into a *Sall* site in pRV5'. The resulting plasmid, RVAΔneo, consisted of both the 5' and 3' flanking sequences and the negative selection marker, HSV-tk. Finally, a 1.9 kb *Eco*RI–*Hind*III fragment that carried a truncated *neo*<sup>r</sup> gene was inserted, by blunt-end ligation, into the single *Sall* site of RVAΔneo. The *neo*<sup>r</sup> gene was under the control of the phosphoglycerate kinase-1 (*pgk*-1) promoter and upstream activating element and was isolated from pKJ-1 (provided by F. Alt, Chen *et al.*, 1993). This resulted in the final construct, RVA, which is diagrammed in Figure 1.

Our initial plan was to use the positive-negative selection scheme devised by Mansour *et al.* (1988) so that we could select against clones that had integrated RVA at random locations within the genome. This selection scheme exploits the fact that DNA replication can be blocked in cells that produce herpes simplex virus thymidine kinase (HSV-tk) through the use of HSV-tk substrate analogs (e.g. gancyclovir and FIAU). As shown in Figure 1, the HSV-tk gene was placed at one end of the RVA recombination vector so that it would likely be retained if the vector integrated randomly within the genome. In preliminary experiments, however, we found that random integration of the HSV-tk gene in 9921 did not render these cells sensitive to either gancyclovir or FIAU. As a result we abandoned this strategy of enriching for homologous recombination events. Others have reported similar difficulty in achieving efficient killing of HSV-tk-expressing cells (e.g. Mombaerts *et al.*, 1991).

### Cell transfections

DNA was introduced into 9921 cells by electroporation (Potter *et al.*, 1984; Potter, 1988). Aliquots of 20 μg of *Xho*I-linearized RVA plasmid DNA were combined with a 1 ml suspension of 10<sup>7</sup> 9921 cells and the mixture dispensed into a 0.4 cm (width) electroporation cuvette (BTX, San Diego, CA). An electric pulse was delivered at 960 μF and 250 V by a Bio-Rad Gene Pulser<sup>TM</sup> electroporator and Capacitance Extender<sup>TM</sup> (Bio-Rad, Hercules, CA). The cells were then diluted in non-selective medium and plated at 10<sup>3</sup> cells/well in 96-well culture plates. After 72 h medium supplemented with 1.5 mg/ml G418 (Gibco-BRL, Grand Island, NY) was added to select for stable transformants expressing the *neo*<sup>r</sup> gene. Colonies were visible 8–16 days after transfection. In most experiments transformants arose in 50–80% of the individual wells on each culture plate.

### Cell fusions

OY21 cells (5 × 10<sup>6</sup>) were fused with equal numbers of 9921Δ3'αE by an electrofusion technique developed in our laboratory (Radomska and Eckhardt, 1996). The two cell types were combined, washed twice in serum-free medium (RPMI) and then resuspended in 0.1 ml of the same serum-free medium. The cell suspension was dispensed into a 0.2 cm electroporation cuvette (BTX, San Diego, CA) and centrifuged at low speed to promote cell-cell contact. Cells then received a 250 V, 960 μF pulse delivered by a Bio-Rad Gene Pulser<sup>TM</sup> electroporator and Capacitance Extender<sup>TM</sup>. After a 60 min incubation at 37°C in a 7.5% CO<sub>2</sub>/air atmosphere the cells were resuspended in 25 ml RPMI 1640 medium containing 10% horse serum and transferred to a 150 × 25 mm tissue culture dish (Catalog No. 08-772-6, Fisher, Pittsburgh, PA). Forty eight hours post-fusion RPMI 1640 medium supplemented with 1 × 10<sup>-3</sup> M ouabain and 1.25 mg/ml G418 was added to the fusion plate to select for hybrids. The OY21 line is resistant to ouabain, but sensitive to G418. The 9921Δ3'αE cell line is sensitive to ouabain, but carries the *neo*<sup>r</sup> gene, making it resistant to G418. Fourteen G418-resistant, ouabain-resistant clones were selected for further analysis.

### Southern blot analyses

Agarose gel electrophoresis, transfer to membrane and DNA hybridizations were performed essentially as described previously (Radomska *et al.*, 1994), with minor modifications. Briefly, ~18 μg restriction enzyme-digested DNA was loaded into each lane of a 0.7% agarose gel. Size-fractionated DNA was then transferred to Nytran (Schleicher & Schuell Inc., Keene, NH). Blots were pre-hybridized and hybridized at 65°C in buffer containing 7.5 × Denhardt's solution, 3 × SSC, 100 μg sonicated salmon sperm DNA and 0.5% SDS. Probes were labeled by the random primer method using a MegaPrime<sup>TM</sup> labeling kit (Catalog No. RPN1605, Amersham, Arlington Heights, IL). For all hybridizations the mouse sequences were separated from vector fragments before labeling and use as radioactive probes.

Probes included pJ11, 3'αE, H3.1, '5' junction fragment' and '3' junction fragment'. pJ11 is a *Bam*HI–*Eco*RI fragment derived from the J<sub>H</sub> gene region of BALB/cJ liver DNA and subcloned into pBR322 (Marcu *et al.*, 1980). 3'αE corresponds to the 596 bp enhancer element previously described by us (Lieberson *et al.*, 1991). H3.1 is a 2.4 kb fragment originally derived from the Ch 9.9.2.1 phage clone and corresponds to the *Hind*III fragment located directly 3' of the 9921 class switch site (Eckhardt and Birshstein, 1985). The 5' junction probe is a 2.8 kb *Sma*I–*Eco*RI fragment that contains the membrane exon of Cα. It was isolated from plasmid IgA3 (a gift of S. Morrison) which contains Cα sequences subcloned into pBR322 (Gregor and Morrison, 1986). The 3' junction probe is a 500 bp *Eco*RI–*Xba*I fragment derived from the 3' end of the 3.7 kb *Xba*I fragment which spans 3'αE (5'→3' position based on natural orientation with respect to IgH gene transcription).

### Northern analyses

Total cellular RNA was isolated by the guanidinium thiocyanate method (Chomczynski and Sacchi, 1987). Approximately 20 μg total RNA/sample were analyzed. RNA samples were denatured with formamide, size-fractionated on 1% formaldehyde-agarose gels and transferred to Nytran essentially as described by others (Maniatis *et al.*, 1982). Blots were hybridized to <sup>32</sup>P-labeled DNA probes at 37°C for ~48 h in a buffer solution of 50% formamide, 7.5 × Denhardt's solution, 5 × SSC, 50 mM NaPO<sub>4</sub>, pH 7.4, 100 μg/ml sonicated salmon sperm DNA and 0.5% SDS. To remove non-specifically bound probe, blots were washed once in 0.1 × SSC, 1% SDS at 37°C for 30 min and an additional four times in the same buffer at 65°C (30 min per wash).

γ2a mRNA levels were assessed by using a cDNA probe that covers most of the constant region of γ2a (pγ2a-10-21; Auffray *et al.*, 1980). This probe detects both γ2a and γ2b transcripts. To verify the integrity of RNA samples and to determine relative mRNA levels among the samples, blots were stripped and rehybridized to a Cκ probe (Cκ; Radomska *et al.*, 1994) which detects Ig κ transcripts.

### Enzyme-linked immunosorbent assays (ELISA)

Microtiter plates (Dynatech Laboratories, Chantilly, VA) were coated with 10 μg/ml affinity-purified Fc fragment-specific rabbit anti-mouse IgG (Catalog No. 315-005-008, Jackson ImmunoResearch Labs, West Grove, PA). Coated wells were then incubated with 50 μl samples of cell culture supernatants or cell lysates. Cell culture supernatants were harvested from 48 h cultures of 1 × 10<sup>6</sup> cells and were serially diluted in the assay. Cell lysates were prepared by lysis of 1 × 10<sup>6</sup> cells in 50 μl 0.5% Nonidet P-40 lysis buffer (Zaller and Eckhardt, 1985). Again, serial dilutions of the lysates were assayed to determine Ig levels.

γ2a heavy chains were detected with alkaline phosphatase-conjugated rabbit anti-mouse γ2a (subclass specific; Catalog No. 315-005-008, Jackson ImmunoResearch Labs, West Grove, PA), using *p*-nitrophenol phosphate as the enzyme substrate (Catalog No. 104 phosphatase substrate tablets, Sigma, St Louis, MO). The absorbance at 405 nm was measured in an ELISA plate reader (Bio-Rad, Hercules, CA). For κ light chain-specific assays plates were coated with 10 μg/ml affinity-purified goat anti-mouse κ antibody (Catalog No. OB1140-UNL, Fisher Biotech, Pittsburgh, PA). Bound κ chain was detected by incubation with biotinylated goat anti-mouse κ antibody (Catalog No. RPN1179, Amersham, Arlington Heights, IL) and subsequent addition of an alkaline phosphatase-avidin conjugate (Catalog No. 43-4422, ZYMED, South San Francisco, CA).

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## Cutting Edge: Ig Heavy Chain 3' HS1-4 Directs Correct Spatial Position-Independent Expression of a Linked Transgene to B Lineage Cells<sup>1</sup>

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Sven Pettersson<sup>3†</sup>

The Ig H chain locus is regulated by a set of *cis*-acting elements. Hypersensitive sites (HS) located 3' of the IgH, HS1-4, has been suggested to act as a locus control region (LCR) in cell lines. To assess the proposed role of HS1-4 acting as an LCR, we generated transgenic mice harboring a V<sub>H</sub> promoter- $\beta$ -globin reporter gene linked to the Ig H chain HS1-4 3' regulatory sequences. Transgene expression is strictly confined to B lymphocytes, with no detectable expression outside the B cell lineage in all transgenic founder lines. Furthermore, reporter gene activity is integration independent but not copy number dependent. Thus, additional sequences are required to allow the HS1-4 regulatory region to act as a classical LCR in mice. Our data are discussed in the context of tissue-specific gene expression in B lineage cells. *The Journal of Immunology*, 1999, 163: 4637-4641.

**R**egulation of transcription and rearrangement in the Ig H chain (IgH)<sup>4</sup> locus is tuned by a complex interplay of multiple regulatory elements. Germline transcription of the V<sub>H</sub> and C $\mu$  region and initiation of VDJ rearrangements are regulated by upstream elements including the V<sub>H</sub> promoter, the DQ52 promoter/enhancer, and the E $\mu$  enhancer (1). However, the problems to direct Ig-gene expression in a correct spatial and temporal fashion have focused the attention on additional regulatory elements located within the 3' end of the IgH locus (2). One of these enhancer elements, the IgH 3' enhancer (3-6) (Fig. 1), has been shown to be active in late B cell development and can be activated in resting B cells in a ligand-receptor-dependent fashion (7, 8).

DNase I hypersensitivity assays and functional assays have revealed three additional transcriptional active enhancers (HS3a, HS3b, and HS4) in addition to the 3' enhancer (HS1,2) in the 3' end of the IgH locus (9-11) (Fig. 1). The overall structure of the IgH 3' region encompassing the HS3a123b4 enhancer elements (referred to as HS1-4) consists of an over 20-kb long palindrome. Thus, HS3a and HS3b elements are part of large inverted repeats flanking the HS1,2 enhancer (12).

A number of Ig-transgenic lines, linked to V<sub>H</sub> promoter-IgH-E $\mu$ , have been generated with high levels of expression in B lymphocytes but always with aberrant expression outside B lineage cells. Similar data have also been observed with the HS1,2 enhancer-driven transgenes (7, 13).

A DNA fragment containing all 3' HS sites except HS3a has previously been shown to direct position-independent and copy-dependent expression of a linked *c-myc* gene that was integrated as a stable transfectant in a plasmacytoma cell line. The authors suggested that the 3' end of the IgH locus might act as a locus control region (LCR) (11). An LCR, as originally identified in the  $\beta$ -globin locus, is defined functionally by its ability to direct tissue-restricted expression of a linked gene in a position-independent, but copy number-dependent, manner (14-17).

To assess the purported role of the IgH 3' region, acting as an LCR, transgenic mice carrying all the HS1-4 3' IgH enhancers were generated. Whereas this region directs correct B cell-specific expression of a linked V<sub>H</sub> promoter-dependent reporter gene, no strict copy-dependent expression is observed. Thus, the mechanism of action of the 3' regulatory region on the IgH locus, and IgH gene expression, is more complex than previously anticipated.

### Materials and Methods

#### Construction of plasmid for microinjection

The pV<sub>H</sub>-LCR vector contains all four 3' IgH enhancers inserted downstream of the reporter gene. The HS1,2 is a 0.6-kb *StuI-EcoRV* DNA fragment (3, 4). HS3a and HS3b are duplicated enhancers flanking the HS1,2 enhancer but orientated in opposite directions on the chromosome. Two 2.1-kb *EcoRI-HindIII* genomic fragments, HS3a and HS3b, respectively, were prepared and inserted on both sides of the HS1,2 enhancer, thus mimicking the endogenous configuration (10, 18). HS4 is the 1.38-kb *PstI-HindIII* DNA fragment (11). The pV<sub>H</sub> promoter is a 0.2-kb *HindIII* fragment derived from a rearranged murine V<sub>H</sub> segment.

#### Generation of transgenic mice

A purified pV<sub>H</sub>-LCR DNA fragment was used to generate transgenic founder lines (7). Positive founders were identified by PCR and Southern

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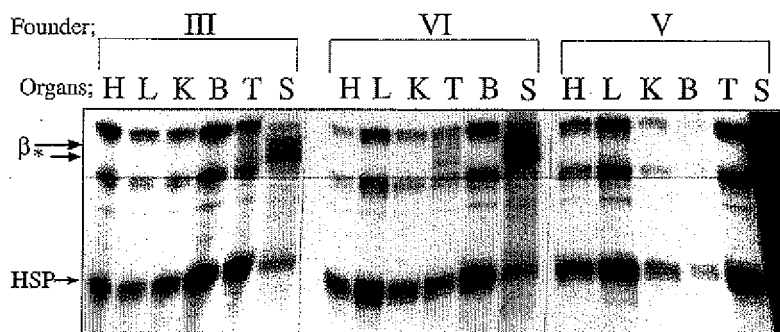
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<sup>4</sup> Abbreviations used in this paper: IgH, Ig H chain; LCR, locus control region; HS, hypersensitive site; HSP, heat shock protein; RPA, ribonuclease protection assay.

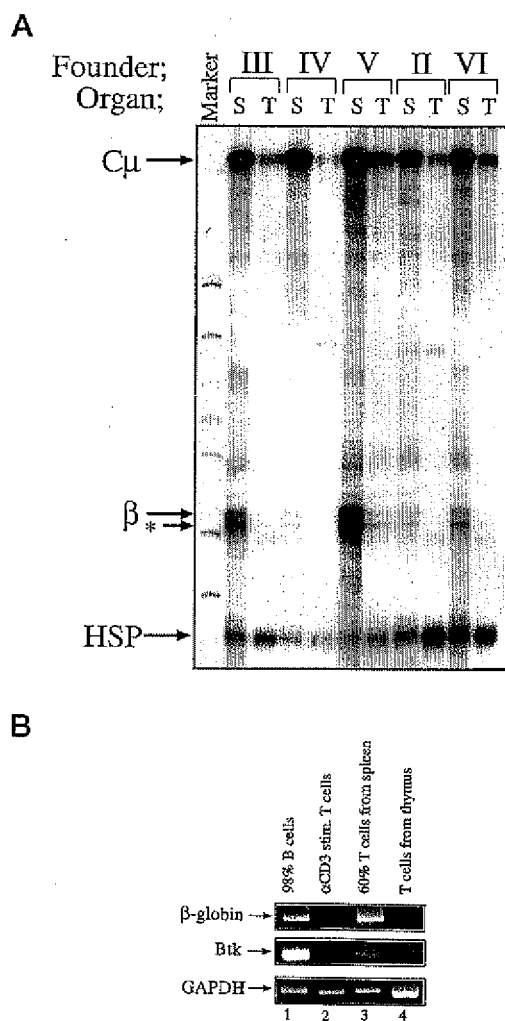


**FIGURE 3.** Expression of the transgene is tissue restricted. Transgenic lines were examined for expression in various organs. RPA showing the expression pattern of the transgene of the three representative transgenic lines. H, heart; L, liver; K, kidney; B, brain; T, thymus; and S, spleen. Arrows show the migration of the specific transcript ( $\beta$ ), the cryptic transcript (\*), and the internal standard (HSP).



examine the expression observed in thymus, we included a  $C\mu$  riboprobe, which detects endogenous  $C\mu$  gene expression in B lymphocytes, and hence indirectly the number of B cells (Fig. 4A). All the thymus RNAs examined contained relatively high levels of the  $C\mu$  transcript (Fig. 4A), and, since T cells express only mar-

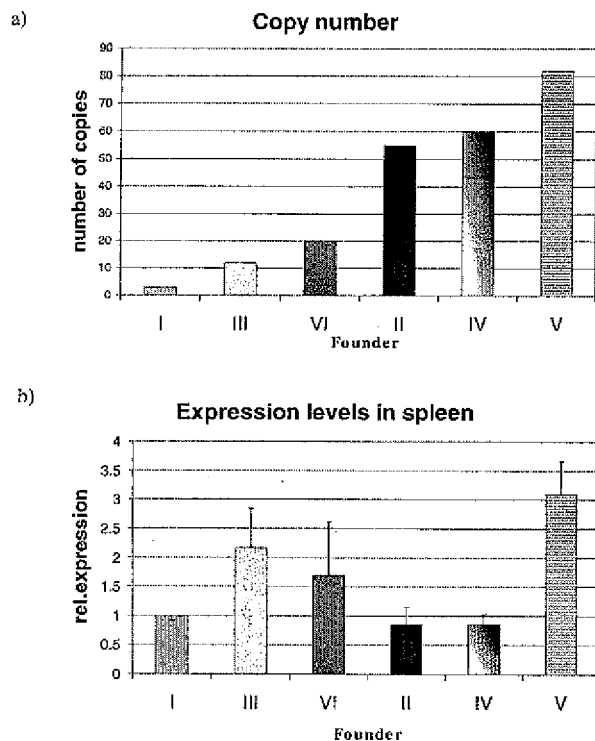
ginal levels of  $C\mu$  (20), we concluded that the  $C\mu$  expression observed originates mainly from activated B cells contaminating the thymus preparations. In addition, RT-PCR analysis was performed, using MACS-sorted splenic B cells as well as T lymphocytes isolated from spleen and thymus originating from the high copy number founder V. The purity of the sorted cells was determined by FACS analysis and found to be 98% for B lymphocytes, 60% for the T cells isolated from spleen, and 99% for thymus (data not shown). To further select for a pure T cell population, Thy1-enriched splenic T cells were stimulated with anti-CD3 for 48 h in vitro. As a control for B cell contamination, we used the expression of the cytoplasmic tyrosine kinase Btk, known to be expressed in B cells but not in T cells (21). GAPDH was used as control for the amount of cDNA. As shown in Fig. 4, expression of the transgene was exclusively found in B lymphocytes or in T cell preparations containing contaminating B lymphocytes, as indicated by the positive signal from the Btk control. Moreover, no transgene expression was detected in the anti-CD3-activated pure T lymphocyte population or in the purified T cells from the thymus, where also no detectable amount of B cell contamination was observed, determined by the absence of a positive Btk signal (Fig. 4). In conclusion, these data show that the  $pV_H$ -driven transgene under the control of HS1-4 is expressed in a stringent tissue-specific manner in B cells.



**FIGURE 4.** Expression of the transgene is restricted to B lineage cells. A, RPA with RNA prepared from spleen (S) and thymus (T) from different founder lines as marked above. Indicated by arrows are the specific transcripts for the  $C\mu$  ( $C\mu$ ) and HSP70 controls (HSP), as well as that of the transgene ( $\beta$ ). B, RT-PCR analysis from MACS-sorted B and T cells. Indicated are the PCR-amplified  $\beta$ -globin product, the Btk product, and the GAPDH product.

#### 3' region and copy-dependent transgene expression

To firmly evaluate whether the HS1-4 enhancers possessed LCR properties, as described in cell lines (11), we examined the expression of the transgene in relation to the copy number. Copy numbers were determined by Southern blot analysis using a hybridization probe covering the  $\beta$ -globin gene to the *EcoRI* site and the complete  $V_H$  promoter. The same blot was subsequently hybridized with a probe specific for HS4 as internal control (data not shown). Data representing the corrected copy number were plotted (Fig. 5A) and shown to range from 2–3 (founder I) to 80–82 (founder V) copies. Transgene expression levels were analyzed by RPA and compared with an internal standard (HSP70). Fig. 5B shows the relative expression of the  $\beta$ -globin reporter gene corrected by the expression of HSP70 and represents the mean value of three independent experiments. Although these experiments show an overall tendency for copy dependence, there is no strict correlation. Particularly, two founder lines (II and IV) with high copy numbers gave rise to only low levels of expression. Furthermore, even though the expression of the transgene was increased in all founders proportionally, upon stimulation of splenic cells with LPS for 72 h in vitro, we did not obtain copy number-dependent expression (data not shown).



**FIGURE 5.** Expression of the transgene is not copy dependent. *a*, Copy numbers of the transgenic founder lines. Copy numbers of the individual founder animals were calculated from the intensity of the bands, quantified by PhosphorImager. Copy numbers were corrected by an internal control and plotted against the number of the founder line. *b*, Expression levels of the transgene measured in spleen. The intensity of the specific transcript (measured by PhosphorImager quantification) of the transgene was divided by the intensity measured from the HSP70 transcript and plotted as relative expression against the number of the founder line. Data represent the mean values of three individual experiments.

## Discussion

Here we show that a  $V_H$  promoter- $\beta$ -globin reporter gene linked to the HS 1-4 of the IgH locus exhibits B cell-specific expression, thus reproducing the endogenous expression pattern of IgH genes. Using RT-PCR analysis, we show that the transgene is expressed only in splenic B cells or impure T cell fractions that contained residual amounts of B cells, but not in pure T lymphocyte preparations from the thymus or anti-CD3-stimulated splenic T lymphocytes. This is the first time that the endogenous expression pattern of the IgH genes is fully reproduced in a transgenic mouse model. Studies on single enhancer elements in transgenic animals have failed to reconstruct the endogenous expression pattern of the Ig genes, and, in all instances, aberrant expression in other organs have been observed additionally to the spleen. Transgenic mice harboring a  $V_H$  promoter/intronic  $E\mu$  enhancer-linked reporter gene will be predominantly expressed in lymphoid cells (B and T) but with detectable expression in other organs (20, 22). This expression pattern was found even in the presence of the matrix attachment regions flanking the  $E\mu$  enhancer, which have been shown to be important for enhancer activity as well as chromatin accessibility (23, 24). Similarly, a  $V_H$ /HS1,2 linked-reporter gene can direct preferential expression to both B and T lineage cells (13). Interestingly, addition of HS3a, HS3b, and HS4 enhancers in the p $V_H$ -LCR construct abrogates the aberrant expression in thymocytes. At present, we do not know the underlying molecular mechanism that directs correct B cell-specific gene expression.

One possibility may be that different transcription factors will bind to the enhancer elements forming a closed structure that could prevent a nonwarranted interaction with other proteins. In the situation with single enhancer elements, such elements would be more accessible to be *trans*-activated by additional transcription factors and perhaps interact with other *cis*-acting elements. Alternatively, active repression could be responsible for the down-regulation of expression in T lymphocytes. Recently, a protein binding to a conserved site in the HS3a and HS3b has been described that forms a complex with small Maf proteins. Bach2 is exclusively expressed at early stages of B cell differentiation and has been suggested to act as a repressor on the IgH 3' enhancer region (25). A similar protein could account for the repression of the IgH genes in thymocytes.

In a study employing a stably transfected human B cell line the integration of a *c-myc* gene under the control of HS1,2,3b,4 was shown to result in a copy number-dependent and position-independent expression (11). To further assess this issue, we have analyzed the  $\beta$ -globin transgene under control of the HS1-4 in transgenic animals. Although high levels of integration-independent transgene expression in all founders were observed, there was not a strict copy number-dependent expression. We can, of course, not exclude that transgene copies, in the high copy number animals, are transcriptionally inactive. However, fluorescence in situ hybridization (FISH) analysis demonstrated that none of the founders had the transgene integrated in an area of heterochromatin (data not shown). This discrepancy between previous data in cell lines (11) and our study may be explained by the fact that the stably transfected clones are drug selected. Only clones with a certain expression level of the selection marker will be chosen and thereby bias the analysis. On the contrary, no selection pressure is installed on transgenic mice, and all founder lines were analyzed. Similar observations have also been made in the analysis of the  $\beta$ -globin locus or the  $E\mu$  enhancer (26, 27). The construct in the plasmacytoma study did not contain HS3a (11), but it appears unlikely that this enhancer alone would account for the difference observed in the present study. The data presented here do not give support for the proposed model of the 6-kb minilocus of the 3' HS1-4 acting as an LCR in a strict sense. Additional elements may be missing in our construct for the completeness of the LCR. The full palindromic structure of the 30-kb endogenous locus centered at the HS1,2 was not reproduced in our animals; in particular, the inverted repeats flanking HS1,2, which were shown to significantly increase the activity of HS1,2 in plasma cells, were incomplete (28) and may contain elements necessary for a copy number-dependent expression. Alternatively, but not mutually exclusively, it is tempting to speculate on a model of a split LCR composed of the  $E\mu$  enhancer and the 3' HS1-4 that surround the Ig genes (see Fig. 1). In the endogenous locus, a cooperation between the intronic and 3' enhancer elements with the  $V_H$  promoter ensures the correct spatial and temporal expression of Ig genes. The absence of  $E\mu$  and its flanking matrix attachment regions may have influenced the LCR function in our animals. Such a model is further supported by the finding that  $E\mu$  together with HS4 is active in early B cell development. Both 5' and 3' elements may therefore be necessary to border and insulate the Ig locus, keeping it in an open chromatin conformation. The p $V_H$ -LCR construct described here has the unique ability to direct B lymphocyte-specific expression, which indicates that the 3' regulatory region certainly plays a role in the control of Ig gene expression. The proposed model of a split LCR guiding IgH gene expression is very important to test since such experiments will be instrumental not only to learn about Ig-gene expression but also to further our understanding on the mechanisms of LCR.

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## Generation of heavy-chain-only antibodies in mice

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Notes:

# Generation of heavy-chain-only antibodies in mice

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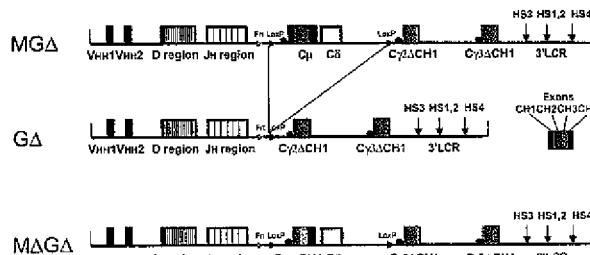
We have generated transgenic mice containing hybrid llama/human antibody loci that contain two llama variable regions and the human D, J, and C $\mu$  and/or C $\gamma$  constant regions. Such loci rearrange productively and rescue B cell development efficiently without LC rearrangement. Heavy-chain-only antibodies (HCAb) are expressed at high levels, provided that the CH1 domain is deleted from the constant regions. HCAb production does not require an IgM stage for effective pre-B cell signaling. Antigen-specific heavy-chain-only IgM or IgGs are produced upon immunization. The IgG is dimeric, whereas IgM is multimeric. The chimeric HCAb loci are subject to allelic exclusion, but several copies of the transgenic locus can be rearranged and expressed successfully on the same allele in the same cell. Such cells are not subject to negative selection. The mice produce a full antibody repertoire and provide a previously undescribed avenue to produce specific human HCAb in the future.

Immunoglobulin rearrangement | transgenic

Conventional antibodies contain two heavy and light chains (LC) coded for by heavy and LC loci. B cell development and antibody production starts in the bone marrow (BM) by heavy chain (HC) VDJ recombination and expression of IgM associated with a surrogate LC on the cell surface. In a second round of recombination, one of the LC rearranges in pre-B cells. If successful, the B cells undergo selection, affinity maturation, and switching to different HC constant regions to result in B cells, which express tetrameric antibodies of different isotypes (IgA, IgG, and IgE). Normally absence of HC or LC expression leads to arrest of B cell development. However, some species produce HC-only antibodies (HCAb) as part of their normal B cell development and repertoire. The best-known HCAb (i.e., no LC) are IgG2 and IgG3 in camelids (1). They undergo antigen-mediated selection and affinity maturation, and their variable domains are subject to somatic hypermutation (2, 3). HCAb are thought to recognize unusual epitopes, such as clefts on the antigen surface (4). The first domain of the constant region, CH1, is spliced out because of the loss of a consensus splice signal (5, 6). CH1 exon loss also has been described in other mammals, albeit associated with disease, e.g., in mouse myelomas (7) and human HC disease (HCD) (8–10).

Camelid HCAbs contain a complete VDJ region. Its size, stability, specificity, and solubility have generated considerable biotechnological interest. The antigen-binding site, a single-variable domain (VHH), resembles VH of conventional Abs. However, differences in FR2 and CDR3 prevent VHH to pair with a variable LC, whereas hydrophilic amino acids provide solubility (11). HCAb of the IgM class have not been found in camelids, suggesting that the IgM<sup>+</sup> stage of HCAb formation is very transient and/or circumvented.

Murine NSO myeloma cells can express a rearranged camelid VHH- $\gamma$ 2a gene (12) and, recently, the same gene was expressed in transgenic mice (13). Here, we describe transgenic mice containing various nonrearranged chimeric HCAb loci and show they rearrange properly, result in allelic exclusion, efficiently rescue B cell development, and undergo class switch recombination and affinity maturation. They generate functional HCAbs after antigenic challenge, providing a previously undescribed way of producing human



**Fig. 1.** The transgenic loci. Two llama VHH exons are linked to the human HC diversity (D) and joining (J) regions, followed by the C $\mu$ , C $\delta$ , C $\gamma$ 2, and C $\gamma$ 3 human genes and human HC Ig 3' LCR. The different constant region exons are shown in different colors (see Middle Right Inset). CH1 (red) was deleted from C $\gamma$ 2 and C $\gamma$ 3 genes in constructs MGA and GA and also from C $\mu$  in construct MΔGA. LoxP sites (in red) enable removal of C $\mu$  and C $\delta$  genes by cre recombination. The Frt site (in green) enables the generation of a single copy from a multicopy array by Flp recombination.

HCAb when the llama VHH regions are replaced with soluble human VH.

## Results

**The CH1 Splice Mutation Is Insufficient for Exon Skipping in the Human HC Locus.** It is not known whether the generation of HCAb (IgG2 and 3) in camelids needs an IgM<sup>+</sup> stage. Hence, we made two hybrid chimeric loci, one locus (MGS) with human C $\mu$ , C $\delta$ , C $\gamma$ 2, and C $\gamma$ 3 constant regions and one with only C $\gamma$ 2 and C $\gamma$ 3 (GS; Fig. 7, which is published as supporting information on the PNAS web site) in a  $\mu$ MT background (14).  $\mu$ MT animals do not produce surface IgM and have a block in B cell development at the pre-B cell stage. The C $\gamma$  regions first were mutagenized to contain the camelid CH1 splice mutation (5). GS was generated because of later reports showing that  $\mu$ MT mice produce some IgG, IgA, and IgE in the absence of membrane IgM (15–17), suggesting some B cells develop without IgM surface expression. Instead of mutating human VH domains to improve solubility (18, 19), two llama VHHs were introduced. Camelid VHH contain characteristic amino acids at positions 42, 49, 50, and 52 (20, 21). VHH1 contained these four, but VHH2 had a Q instead of an E at 49. The locus contained all of the human HC D and J regions and the locus control region (LCR) (Fig. 7). Surprisingly, the splice mutation gave incorrect CH1 exon skipping in mice and no chimeric Ig expression (Fig. 7).

**Author contributions:** R.J., F.G., and D.D. designed research; R.J., S.D., R.W.H., A.v.R., and D.D. performed research; G.P. and J.K.-a.S. contributed new reagents/analytic tools; R.J., S.D., R.W.H., G.P., F.G., and D.D. analyzed data; and F.G. and D.D. wrote the paper.

The authors declare no conflict of interest.

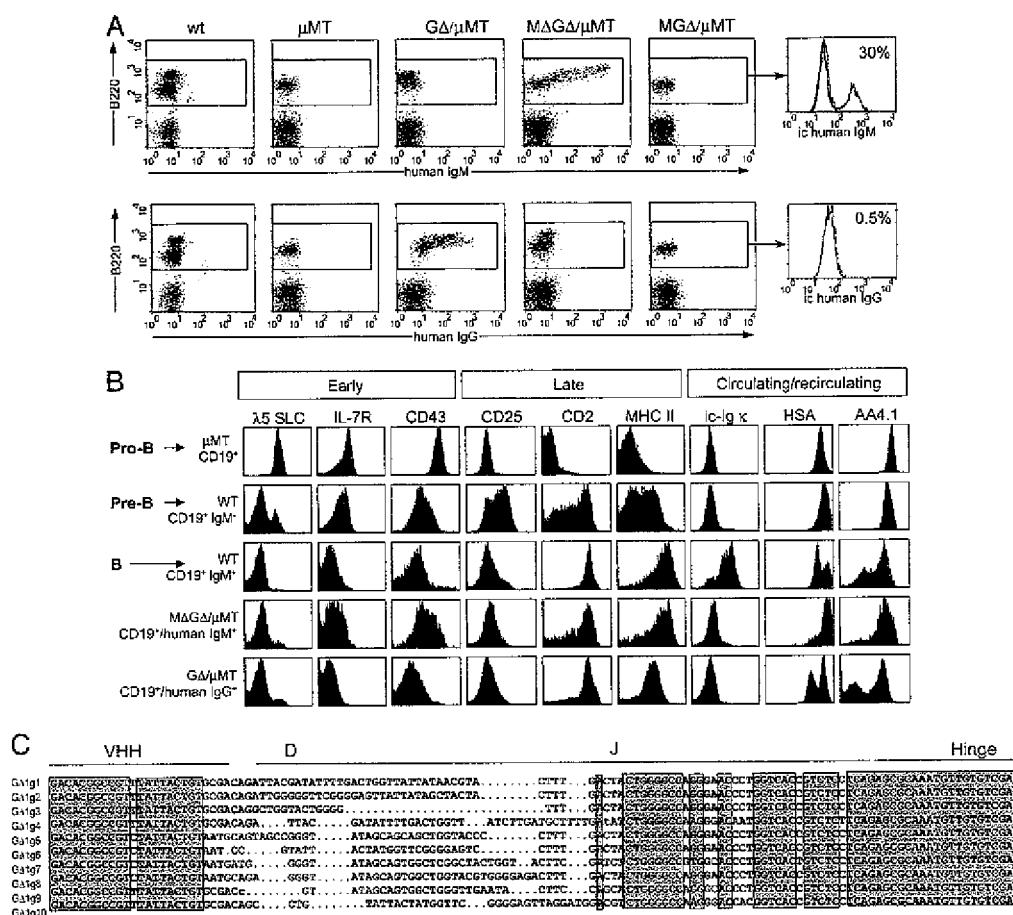
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**Abbreviations:** BM, bone marrow; HC, heavy chain; HCAb, HC-only antibody; LC, light chain; sdAb, single-domain antibody.

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**Fig. 2.** Flow cytometric analysis of B cells of wt,  $\mu$ MT, MGA/ $\mu$ MT, M $\Delta$ G/ $\mu$ MT, and G $\Delta$ / $\mu$ MT mice in BM. (A) Lymphoid cells were gated on forward and side scatter. Surface expression of B220 and chimeric IgM or IgG is shown as dot plots. For MGA/ $\mu$ MT, the B220<sup>+</sup> fraction was gated and analyzed for the expression of intracellular (ic) chimeric Ig  $\mu$  and  $\gamma$  H chains, displayed as histogram overlays (red lines), with background stainings of B220<sup>+</sup> cells from  $\mu$ MT mice (black lines) as controls. The % of positive cells is indicated. (B) M $\Delta$ G or G $\Delta$  transgenes rescue pre-BCR and BCR function. Shown are the expression profiles of the indicated markers in total CD19<sup>+</sup> fractions from  $\mu$ MT mice (pro-B cells), in CD19<sup>+</sup> surface IgM<sup>+</sup> fractions (pro-B/pre-B cells), and CD19<sup>+</sup> surface IgM<sup>+</sup> fractions (B cells) from WT, M $\Delta$ -G $\Delta$   $\mu$ MT, and G $\Delta$   $\mu$ MT mice. ic-Ig  $\kappa$ , intracellular Ig  $\kappa$  LC. Flow cytometric data are displayed as histograms representative of 3–8 animals examined in each group. (C) Sequence alignment of BM cDNA showing VDJ recombination. Sequences are from G $\Delta$ . Green shows sequence identity.

**Chimeric Loci Lacking a Human CH1 Region.** The CH1 splice problem was solved by generating three new constructs (Fig. 1), all containing C $\gamma$ 2 and C $\gamma$ 3 with CH1 deleted, one with C $\mu$  and C $\delta$  (MGA), one without C $\mu$  and C $\delta$  (G $\Delta$ ), and one with CH1 deleted C $\mu$  (M $\Delta$ G $\Delta$ ). Three MGA, six G $\Delta$ , and four M $\Delta$ G $\Delta$  transgenic mouse lines with one to five copies were obtained in a  $\mu$ MT background. Mice with different copy numbers gave the same results.

**G $\Delta$  and M $\Delta$ G $\Delta$  rescue B cell development.** G $\Delta$  and M $\Delta$ G $\Delta$ , but not MGA, rescued B cell development in a  $\mu$ MT background. The rescue of B220/CD19 cells was between 30% and 100% in different lymphoid compartments independent of copy number (Fig. 2A and Table 1). The M $\Delta$ G $\Delta$  mice contain human IgM-producing cells in the BM absent in WT or  $\mu$ MT mice. Appropriately, they have not switched class because chimeric IgG is absent. The G $\Delta$  mice contain only chimeric IgG<sup>+</sup> B cells. The MGA mice contain very few B cells expressing cell-surface chimeric Ig, but interestingly, 30% of the BM B220 cells express intracellular IgM, but not IgG (Fig. 2A). The MGA, but not the M $\Delta$ G $\Delta$  and G $\Delta$  (data not shown), express mouse Ig LC (see Fig. 5G). Thus, the C $\mu$  and C $\gamma$  genes are expressed, and absence of CH1 is crucial for surface-expressed HCAB.

**HCAB replace mouse (pre-)BCR in the BM.** During progression of large cycling into small resting pre-B cells, specific surface markers are down-regulated in a pre-BCR-dependent manner (22). To test whether chimeric HCABs functionally replace the pre-BCR, various markers were analyzed. Pro-B cells express high cytoplasmic SLC, IL-7R and CD43, which are down-regulated upon pre-BCR expression and absent in mature B cells (Fig. 2B).

MGA/ $\mu$ MT or G $\Delta$ / $\mu$ MT chimeric Ig<sup>+</sup> B cells are SLC- and

**Table 1.** Percent of B220<sup>+</sup>/CD19<sup>+</sup> cells in total population of nucleated cells

Cell type	WT	G $\Delta$ (–5 copies)	G $\Delta$ (single copy)	M $\Delta$ G $\Delta$
BM	10.80 $\pm$ 2.09	5.94 $\pm$ 1.44	4.93 $\pm$ 1.79	6.06 $\pm$ 1.53
Spleen	41.80 $\pm$ 6.05	32.14 $\pm$ 9.46	28.70 $\pm$ 8.70	33.95 $\pm$ 3.24
Blood	43.72 $\pm$ 7.50	16.00 $\pm$ 5.68	16.01 $\pm$ 3.76	9.25 $\pm$ 3.24
Peritoneum	21.92 $\pm$ 9.90	22.85 $\pm$ 6.71*	22.30 $\pm$ 7.29*	21.21 $\pm$ 14.42

Mice were 14–20 weeks old. Numbers of mice analyzed are 5–11 per mouse line with the exception of two peritoneal cell measurements, where calculations are based on two samples (marked by asterisks).

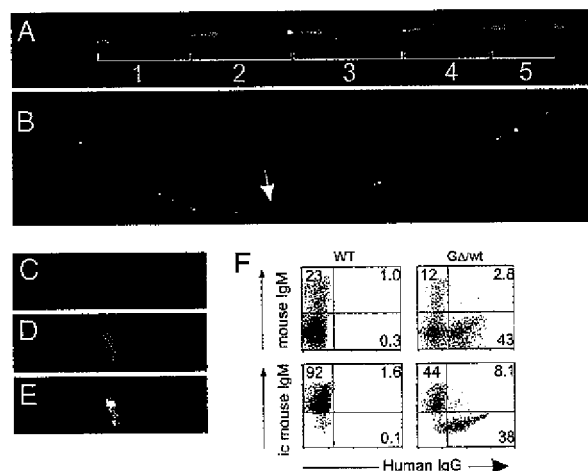
IL-7R-low, indicating that the chimeric HC IgG and IgM receptors function as a pre-BCR in down-regulating SLC and IL-7R. CD43 persists in  $\Delta\Delta$  (not in  $\Delta$ ) mice, perhaps due to increased B-1 B cell differentiation. CD2 and MHC class II are induced normally. The levels of the IL-2R/CD25, transiently present in pre-B cells, are very low on mature  $\Delta\Delta$  or  $\Delta/\mu$ MT B cells as in WT (Fig. 2B).  $\text{Ig}\kappa$  was absent in mature  $\Delta\Delta$  or  $\Delta/\mu$ MT B cells (Fig. 2B) and was not induced in BM cultures upon IL-7 withdrawal after IL-7<sup>+</sup> culture (data not shown). Finally, the chimeric HCab<sup>+</sup> B cell populations in  $\Delta\Delta$  or  $\Delta$  mice consisted of cells generated in the BM (HSA<sup>high</sup> and AA4.1/CD93<sup>high</sup>) and cells matured in the periphery that are recirculating (HSA<sup>low</sup> and CD93<sup>low</sup>) as in wild type.

Thus, chimeric HCab IgG and IgM function as (pre-)BCR with respect to developmentally regulated markers. IgL chain is not induced (see below). Both VHJs are used for VDJ recombination, CH1 is absent and, importantly, CDR3 shows a large diversity (Fig. 2C).

**Multiple Rearrangements and Allelic Exclusion.**  $\Delta\Delta$  and  $\Delta$  hybridomas were made after immunization. Particularly, the five-copy  $\Delta$  line1 could have more than one rearrangement. Of the five different five-copy hybridomas, one rearranged one in frame copy; two hybridomas had two rearrangements, each with one out of frame; one hybridoma had two in-frame rearrangements; and one hybridoma had four rearrangements, with two in frame.

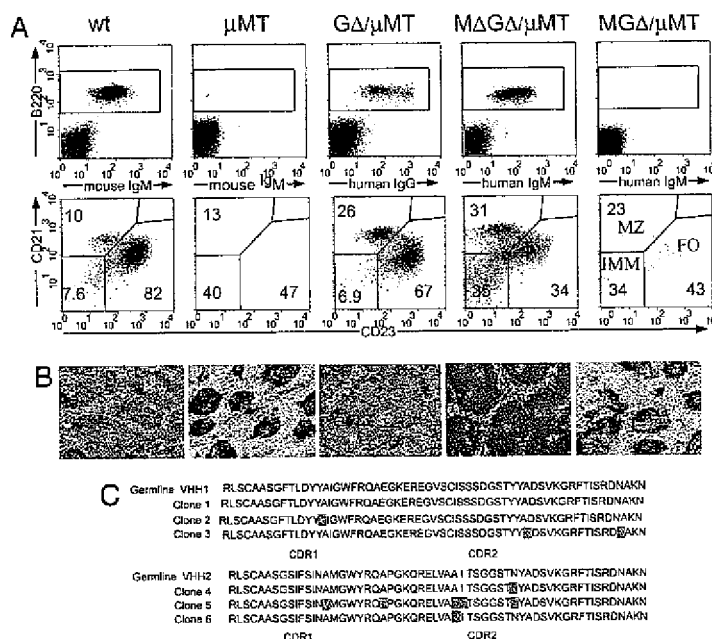
Two express two productive mRNAs (mass spectrometry confirmed the secreted HCabs matching the cDNA; data not shown). We also carried out DNA fiber FISH on a hybridoma with one rearrangement and normal FISH on one with four rearrangements by using an LCR probe detecting each copy and a probe between VHH and D detecting only nonrearranged copies (Fig. 3A–E). Control cells showed five copies plus half a copy at each end (Fig. 3A), in agreement with Southern blots (data not shown), whereas the hybridomas show one and four rearranged copies, respectively (Fig. 3B–E). Thus, multiple copies can rearrange successfully on the same allele.

Moreover B220/CD19-positive BM cells of  $\Delta$  line1 transgenic mice in a WT background were analyzed for the expression of



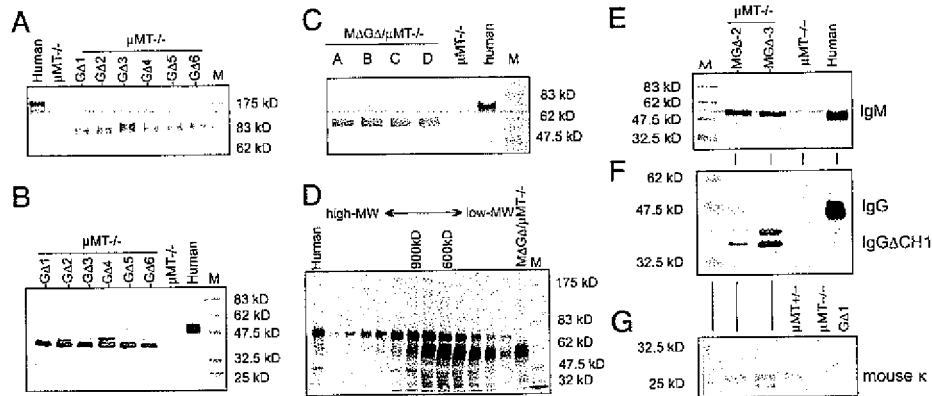
**Fig. 3.** DNA FISH and allelic exclusion of a five-copy chimeric  $\Delta$  locus. (A) Stretched chromatin fiber from lung cells of  $\Delta$  line1 carrying five intact copies (1–5) of the  $\Delta$  locus, flanked by half of a locus containing the LCR (red) and half of a locus containing the VHH to J region (green). (B) Stretched chromatin fiber FISH of a hybridoma (G20) derived from  $\Delta$  line1 B cells where one copy has rearranged (white arrow). (C) Nonstretched DNA FISH of hybridoma T1 with the LCR probe (red). (D) Same as C with a probe between VHH and D (green). (E) Overlay of C and D. Note that T1 has four rearrangements visible because of the loss of four green signals with no loss of red signals. (F) Allelic exclusion in  $\Delta$  mice. Flow cytometric analysis of murine surface or intracellular (ic)  $\mu$ H chain and chimeric IgG on total BM CD19<sup>+</sup> cells from  $\Delta$  transgenic mice in a WT background and a nontransgenic WT control mouse displayed as dot plots. The % of cells within the quadrants is indicated. The average extracellular and intracellular double expressors after subtraction of the background were 1.5% and 5.8%, respectively ( $n = 9$ ).

transgenic IgG and mouse IgM. Clearly, the  $\Delta$  B cells express either mouse Ig or chimeric Ig (Fig. 3F), showing allelic exclusion. **Splenic B cells.** Splenic B cell subpopulations were analyzed by using CD21/CD23 (Fig. 4A).  $\Delta$  CD21<sup>low</sup>CD23<sup>low</sup> immature B cells were



**Fig. 4.** B cell populations in the spleen of WT,  $\mu$ MT,  $\Delta$ ,  $\Delta\Delta$ , and  $\Delta\Delta/\mu$ MT mice. Data shown are representative of 4–8 mice examined in each group. (A Upper) FACS data of spleen cells, stained for mouse IgM, chimeric IgG, chimeric IgM versus B220. (A Lower) Flow cytometric analysis of B cell populations in spleen. Lymphoid cells were gated on forward and side scatter. Surface expression of B220 and the indicated ig (A Upper) or the CD21/CD23 profile is displayed as dot plots and the % of cells within the indicated gates are given. CD21<sup>low</sup>CD23<sup>low</sup>, immature B cells; CD21<sup>high</sup>CD23<sup>low</sup>, marginal zone B cells; CD21<sup>high</sup>CD23<sup>high</sup>, follicular B cells. (B) Histology of the spleen of WT,  $\mu$ MT,  $\Delta$ ,  $\Delta\Delta/\mu$ MT,  $\Delta\Delta$ , and  $\Delta\Delta/\mu$ MT mice. Immunohistochemical analysis is shown after 5- $\mu$ m frozen sections were stained with  $\alpha$ B220 (blue) for B cells and  $\alpha$ CD11c/N418 (brown) for dendritic cells. Arrow indicates a small cluster of B cells in MGA spleen. (C) Sequence alignment of Peyer's patches cDNA showing that the transgenic locus undergoes hypermutation in the CDR1 and 2 regions. Sequences are from the transgenic locus  $\Delta\Delta$  with a CH1 deletion.

**Fig. 5.** Prot G or concanavalin purified serum samples of six different GA lines (A and B), four MΔGA lines (C), and two MGA lines (E–G) in the  $\mu$ MT background run under nonreducing (A) and reducing conditions (B–G). The size of the chimeric IgG (B and F) and IgM (C and D) is consistent with a CH1 deletion and absence of LC. Mouse  $\kappa$  LC were normal size (G). Human serum was used as a positive control. (D) Superose 6 size fractionation of MΔGA serum after mixing in a human IgM control under nonreducing conditions. Each fraction was analyzed by gel electrophoresis under reducing conditions. Fractions collected of the column are from left (high MW) to right (low MW). Controls are human serum alone (first lane) and mouse serum before mixing in the human IgM control serum (lane MΔGA serum). Size markers are indicated.



in normal ranges, and chimeric HC-IgG<sup>+</sup> cells differentiated into follicular (FO; CD21<sup>+</sup>CD23<sup>+</sup>) and marginal zone (MZ; CD21<sup>high</sup>CD23<sup>low</sup>) B cells. In MΔGA, the immature B cells were increased, i.e., differentiation of HC-IgM expressing cells into FO and MZ B cells appear somewhat impaired. CD23 reduction was accompanied by increased CD43 and CD5 (data not shown), indicative of differentiation into B-1 B cells. The few chimeric IgM expressing B cells (also expressing mouse LC, see Fig. 5) in MGA mice had a FO/MZ distribution similar to MΔGA mice.

Spleen architecture in MΔGA and GA, but not MGA mice, is normal (Fig. 4B). As in wild type, germinal centers in B cell follicles are formed (data not shown) during T cell-dependent responses that in GA mice contain chimeric IgG<sup>+</sup> cells. We confirmed hypermutation of the HCAb by cDNA analysis from B cells present in Peyer's patches (Fig. 4C). Both VHs are used. Thus, in GA and MΔGA mice, immature B cells migrating from BM differentiate into spleen FO and MZ B cells and undergo somatic hypermutation upon antigen challenge.

**Single-copy loci rescue efficiently and CH1 absence is essential.** The GA line1 mice (Table 1) had five copies and, hence, the efficient rescue was related possibly to the copy number of the locus. A single-copy line generated from the GA line1 through breeding with a FlpeR line (23) gave the same B cell rescue (Table 1; Fig. 8, which is published as supporting information on the PNAS web site).

Confirmation that a single copy of the locus is sufficient for rescue and that a CH1 region is inhibitory was obtained by cre-mediated deletion of the C $\mu$  and C $\delta$  from MGA line 3, resulting in a single-copy GA line (Fig. 8). This locus now rescues B cell development like the other GA lines. Thus, a CH1 region in C $\mu$  inhibits B cell rescue, and copy number is not important.

**Mouse Light Chains Do Not Rearrange in MΔGA and GA Mice.** Murine LC were absent in the MΔGA and GA mice by Western blots (data not shown, but see Fig. 2B and 5A) or FACS, suggesting that the LC genes do not rearrange as confirmed by comparing the Ig $\kappa$  locus germ-line signals in sorted splenic B220<sup>+</sup> cells and liver DNA by Southern blots (Fig. 9, which is published as supporting information on the PNAS web site). Mouse LC remain in a germ-line configuration. In contrast, LC are present in the few chimeric Ig<sup>+</sup> cells in the MGA/ $\mu$ MT mice (see Fig. 5G).

Thus, the chimeric HCAb expression in early B cell development in BM fails to signal for LC rearrangement. In this respect, HCAb lacking CH1 mimic a BCR rather than a pre-BCR, probably because of a failure to bind pseudo-LC (24).

**Serum analysis.** Chimeric IgM was present in MΔGA and chimeric IgG in both MΔGA and GA serum. In nonimmunized adults, the chimeric IgM ( $\approx 50$   $\mu$ g/ml) and IgG (200–1,000  $\mu$ g/ml) are present

at levels comparable with those seen in WT or mice with a normal human IgH locus (25). All six GA mice had HCAb IgGs with a molecular mass of  $\approx 80$  kDa under nonreducing and  $\approx 40$  kDa under reducing conditions, consistent with HC dimers lacking a LC and each HC lacking CH1 (11 kDa shorter than the control human IgG; Fig. 5A and B).

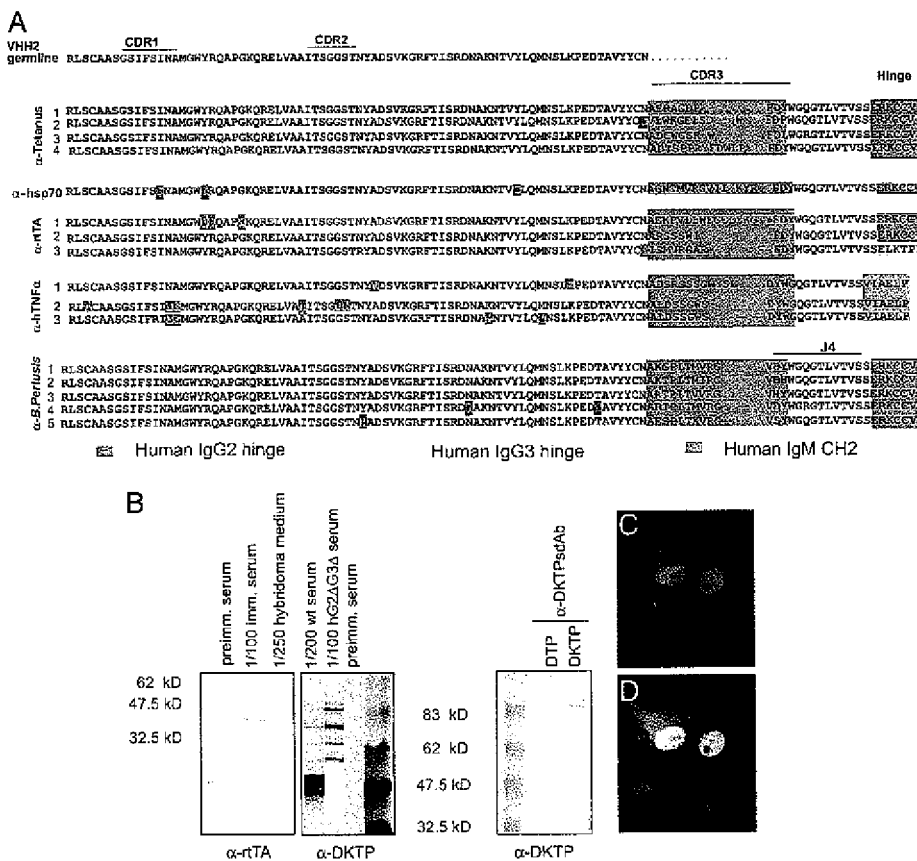
The MΔGA serum had multimeric HC-IgM. Under reducing conditions (Fig. 5C), all four lines had IgM with the molecular mass of a human IgM after subtraction of CH1. Serum also was fractionated (Fig. 5D, horizontal fractions) under nonreducing conditions, and each fraction was analyzed under reducing conditions (Fig. 5D, vertical lanes). When compared with the human pentameric 900-kDa IgM, the transgenic IgM is 600 kDa, consistent with a multimer lacking LC and CH1. Thus, MΔGA or GA mice produce multimeric IgM and/or dimeric IgG.

**MGA Mice.** Some clustered B220-positive cells (<1% of the WT) are seen in MGA/ $\mu$ MT spleens (Fig. 4B), and serum chimeric IgM and IgGs were detected only after purification (Fig. 5E and F). The IgM in these mice was normal size, whereas the IgGs are shorter because of CH1 deletion. Interestingly mouse  $\kappa$ LC, presumably associated with the chimeric IgM, also were detected (Fig. 5G).

**Immunization.** The GA/ $\mu$ MT mice were immunized with *Escherichia coli* hsp70, DKTP (*Diphtheria* toxoid, whole cell lysate of *Bordetella pertussis*, *Tetanus* toxoid, and inactivated poliovirus types 1–3), and rTA (26), the MΔGA mice with human TNF $\alpha$ . Antibodies were isolated from hybridomas or single-domain Ab (sdAb) phage display libraries.

Sequencing (Fig. 6A) showed that both IgG2 (seven of eight) and IgG3 [one of eight] were produced (the sdAb were isolated from a IgG2 library). Different D and J regions were used. When comparing all 14 antibodies, it was evident that all J regions are used, but as in humans, JH4 is used most frequently. Surprisingly, all antibodies had VH2 (with a Q rather than E at position 49; ref. 20). Clearly CDR3 provides most diversity (27). It varies between 10 and 20 aa (average of 13.6 aa), as in llamas and humans (28, 29). Although not at high frequency, the VHH were hypermutated. The 3 $\alpha$ -hTNF $\alpha$  antibodies (Fig. 5) had different hypermutations in the CDR2 region.

The HCAb are functional in regular assays as hybridoma supernatants and bacterial periplasmic fractions of sdAbs (Fig. 6). All were positive in ELISAs and in antigen detection on Western blots (Fig. 6B). We also tested the  $\alpha$ -rTA IgG in immunocytochemistry in a rTA<sup>+</sup> cell line (Fig. 6C and D). The avidity of a number of the antibodies was high, although some were low. For example, binding studies of the  $\alpha$ -rTA antibody used in the immunocytochemistry



**Fig. 6.** Antibody properties. (A) Sequences of monoclonal antibody cDNAs specific for tetanus toxoid; HSP70, rTA, and human TNF $\alpha$ . The top sequence is the germ-line VHH2 sequence. The CDR 1, 2, and 3 and hinge regions are indicated above the sequence. Different isotypes and classes are indicated by different colors on the right. The J regions that are used are indicated on the right. (B) Examples of Western blots by using the different HCab (hybridomas, sera, and sdAb). (B Left)  $\alpha$ rTA serum and hybridoma medium, diluted 1/100 and 1/250. (B Center)  $\alpha$ DKTP serum from WT and GA mice diluted 1/200 and 1/100. (B Right)  $\alpha$ B. pertussis sdAb against vaccine containing B. pertussis antigen (DKTP) or lacking it (DTP), because we were unable to purchase purified B. pertussis antigen. (C and D) Immunostaining of a Tet<sup>+</sup> on cell line with a marker plasmid responding to rTA by expressing a marker in cytoplasm (44). (C) Nuclei expressing rTA (green). (D) Doxycycline-induced expression of the marker (red) in response to rTA and DAPI nuclear staining (blue).

(Fig. 6 C and D) by surface plasmon resonance analysis showed an association rate  $k_{on}$  of  $(2.1 \pm 0.002) \times 10^5$  ( $M^{-1}s^{-1}$ ) and a dissociation rate  $k_{off}$  of  $(4.1 \pm 0.02) \times 10^{-4}$  ( $s^{-1}$ ) resulting in a dissociation constant  $K_D$  of 1.9 nM for anti-rTA.

## Discussion

Here, we reported that modified HCab loci produce HCab and rescue B cell development in  $\mu$ MT mice. Lack of CH1 is crucial for HCab secretion, but the camelid splice mutation at the 3' CH1 border (30) is insufficient for CH1 removal, thus more than this point mutation is required, at least in the human-type locus. VHH-IgM with CH1 blocks B cell development, probably because of ineffective assembly of surface IgM as a pre-BCR. In contrast, mice expressing a HC disease-like human  $\mu$  protein develop normal CD43<sup>+</sup> pre-B cells in a SCID background independent of  $\Delta$ 5 (9). Truncated IgM expressed on the B cell surface without L chains mimics pre-BCR signaling through self-aggregation (10).

Normally BiP chaperones the folding and assembly of antibodies by binding CH1 until it is replaced by (surrogate) LC (31). Although VpreB1 and 2 can bind normal IgM in the absence of  $\Delta$ 5 (32), our results suggest that transgenic  $\mu$  HC pairing with the Vpre protein does not take place when a CH1-containing  $\mu$  HC is linked to a VHH, which would lead to a failure of B cell development (33). The MGS and MGA transgenic mice containing a CH1 would be able to form some pre-BCR-like complex that may lead to signaling, causing some expansion and developmental progression and explain why 30% of the B220-positive cells in BM have intracellular IgM (Fig. 2A). The few matured B cells in spleen of these mice may be explained by the recently described novel receptor complex lacking any SLC or LC (34).

When IgM is removed and Cy 2 and 3 lack CH1, there is rescue of B cell development, showing that IgG can functionally replace IgM. IgG1 expression from the pro-B cell stage onwards, was shown to substitute for IgM in Rag2<sup>-/-</sup> B cell development (35). Recently, it also was shown that a prearranged camelid IgG2a partially rescues B cell development in one transgenic line in a  $\mu$ MT (and a CA<sup>-/-</sup>) background (13). In our case, IgM or IgG lacking CH1 rescue B cell development in 10 of 10 independent lines. Moreover, we see no LC rearrangement and conclude that LC are not required for further B cell differentiation. The difference in our results and those of Zou *et al.* (13) may be explained by the level of expression of the locus (and, thus, signaling) because of the LCR on our constructs. Our results confirm that truncated  $\mu$  HCab lacking CH1 (24) or VH and CH1 (36) cannot associate with SLCs and fail to activate  $\kappa$  gene rearrangement.

Interestingly, one or more HC rearrangements occur in multicopy loci (Fig. 3). Two of the hybridomas, originating from two separate splenocytes gave two productive HCab, confirming that expression of two antibodies in one B cell is not toxic (37). However, the prediction (37) that they would loose in competition with single antibody-producing cells under antigen challenge is not borne out by finding two double antibody-expressing cells of five hybridomas.

The (multicopy) locus is subject to and exerts allelic exclusion in WT background, because BM cells express either mouse or chimeric cell surface Ig. Few BM cells expressed both on the surface (Fig. 3F). Interestingly, a five-copy GA WT mouse has two mouse alleles with one Ig locus each and one allele with five chimeric HCab loci. If alleles are chosen, there should be more mouse than chimeric Ig expression, and if genes are chosen, there should be more chimeric than mouse Ig expression. In fact, mouse Ig is

expressed more often (44/38; Fig. 3F). Ignoring possible deviations from the random V use and a possible position effect on the transgenic locus, suggesting that the first choice is one of alleles.

Normally, a productive rearrangement down-regulates recombination to prevent rearrangement of the other allele. However, the multiple transgenic copies, when rearranged, exclude the mouse endogenous locus, but fail to exclude further rearrangement on the same open locus before RAG down-regulation. This process may involve a spatial component ("compartment"), in that the time before the RAGs are down-regulated would be sufficient to rearrange another gene in the locus because it would be in close proximity. The observation that other species with multiple loci on the same chromosome have more cells expressing two Abs (38) supports this argument. Alternatively multiple rearrangements may take place at the same time.

Importantly, we show that HCAb loci can be expressed successfully in mice. Antigen challenge results in antigen-specific chimeric HCAb of different classes (dependent on locus composition) expressed at levels comparable with WT or conventional human IgH transgenic mice (25). Only two VHHs were used, yet antibodies with diverse specificity were isolated successfully to almost all of the totally unrelated proteins we tested, demonstrating the efficiency and efficacy of diversity generated by CDR3 (27). Thus, having V(D)J recombination and *in vivo* selection provides an advantage over antibodies of fragments thereof from synthetic libraries. Hybridomas containing HCAb with a human effector function are generated easily. They can be used also for direct cloning and expression of sdAb, which can alternatively also be derived by phage display.

Thus, these mice open up new possibilities to produce human HCAb for clinical or other purposes, particularly in light of the evidence (4) that HCABs may recognize "difficult" epitopes such as enzyme active sites. The restricted number of VH may explain why not all antigens were recognized; the polio and *Diphtheria* proteins gave no response in GA mice, whereas WT control mice did (data not shown). Surprisingly, all antibodies had VHH2 lacking a conserved amino acid (39) at position 49 in contrast to VHH1 that has one and should be more soluble. Perhaps, VHH1 expression results in negative selection.

The addition of more VHs should lead to an even broader repertoire. Whilst it is preferable to avoid multiple copies on a single allele, it would be advantageous to have multiple alleles with a single copy of different VH regions to increase diversity. In such new loci,

one can use either normally occurring (human) VH or VH engineered for increased solubility (18).

In conclusion, we show that antigen-specific HCAb of potentially any class can be produced in mice. By introducing soluble human VH domains in the locus, this technology allows the production of fully human HCAb of any class or fragments thereof in response to antigen challenge for use as therapeutic agents in man. By using different vertebrate loci, our technology also allows for production of antibodies from any vertebrate for use as reagents, diagnostics, or for the treatment of animals.

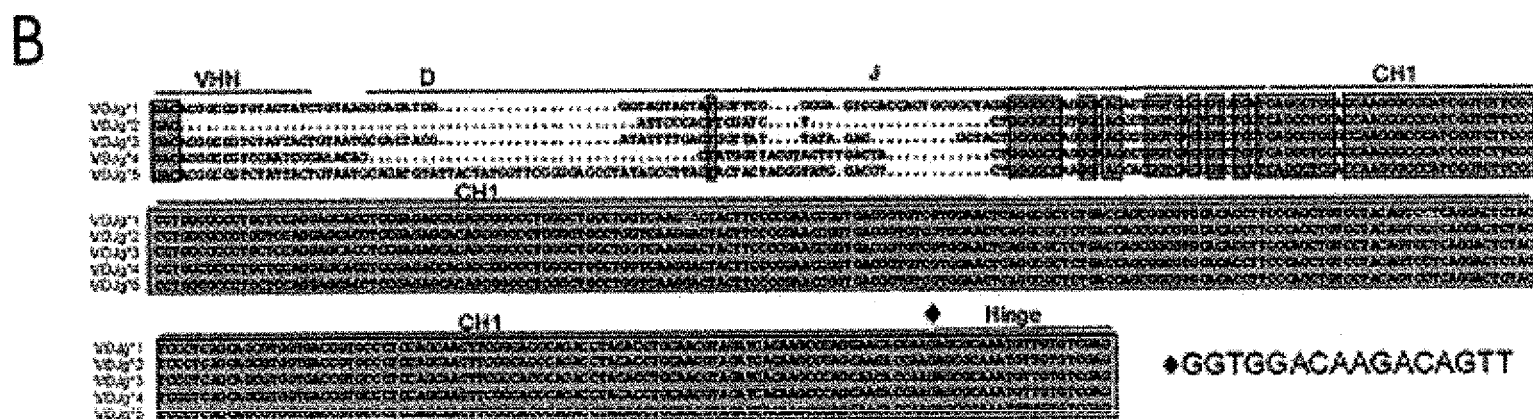
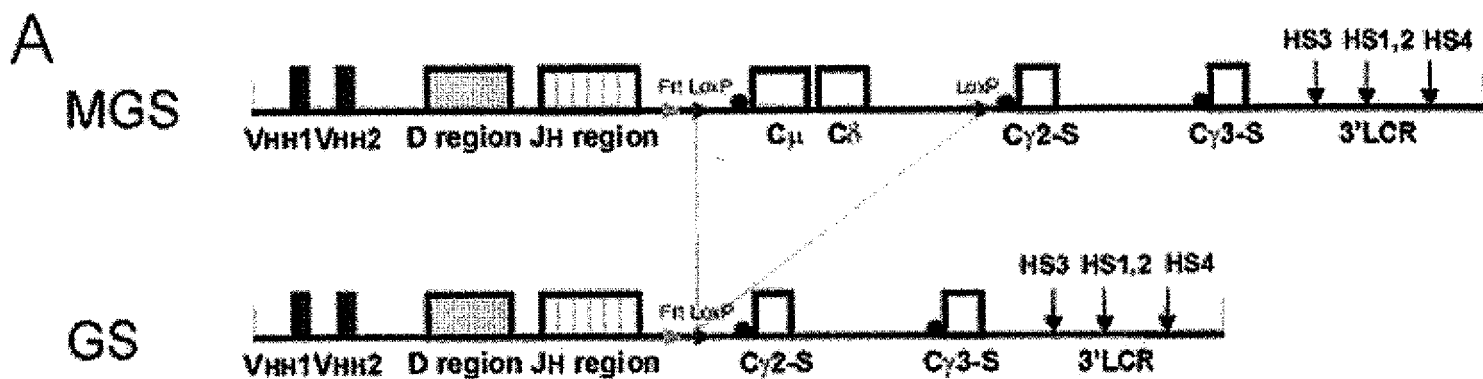
## Materials and Methods

A standard genomic cosmid library was made from *Lama glama* blood. Two germ-line VHHs were chosen with hydrophilic amino acid codons at positions 42, 50, and 52 according to ImmunoGeneTics numbering (40), one with and one without a hydrophilic amino acid at 49. One is identical to IGHV1S1 (GenBank accession no. AF305944), and the other has 94% identity with IGHV1S3 (GenBank accession no. AF305946). PAC clone 1065 N8 contained human HC D and J regions, C $\mu$  and C $\delta$ , and clone 1115 N15 contained C  $\gamma$ 3 (BACPAC Resource Center, Oakland, CA). Bac clone 11771 (Incyte Genomics, Palo Alto, CA) was used to obtain C $\gamma$ 2 and the HC-LCR (41). C $\gamma$ 3 and C $\gamma$ 2 were subcloned separately into pFastBac (Invitrogen, Carlsbad, CA). The point mutation (G to A) (5) or deletion of CH1 was done by recombination (42). Similarly, *frt* and *lox P* sites were introduced 5' to the C $\mu$  switch region, and a second *lox P* site was placed 5' to the C $\gamma$ 2 switch region, resulting in MGS or MGA.

GS or GA were generated from MGS or MGA (Figs. 1 and 7) by cre recombination (43). MGA was obtained from MGA by deletion of the C $\mu$  CH1 region through homologous recombination. The generation of transgenic mice, breeding, and genotyping, RT-PCR, flow cytometry, Ig gene arrangement, DNA FISH analysis, immunization and hybridoma production, sdAb library production and screening, immunocytochemistry, Western blots, gel filtration, and BIAcore measurements are described in *Supporting Methods*, which is published as supporting information on the PNAS web site.

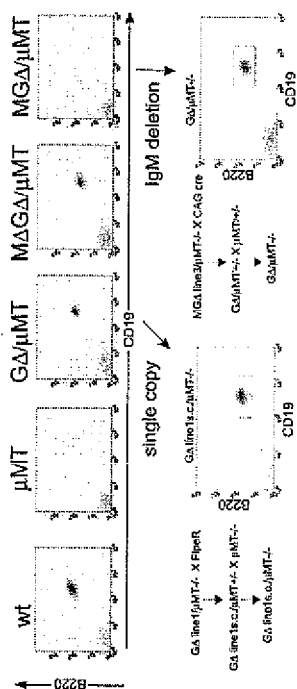
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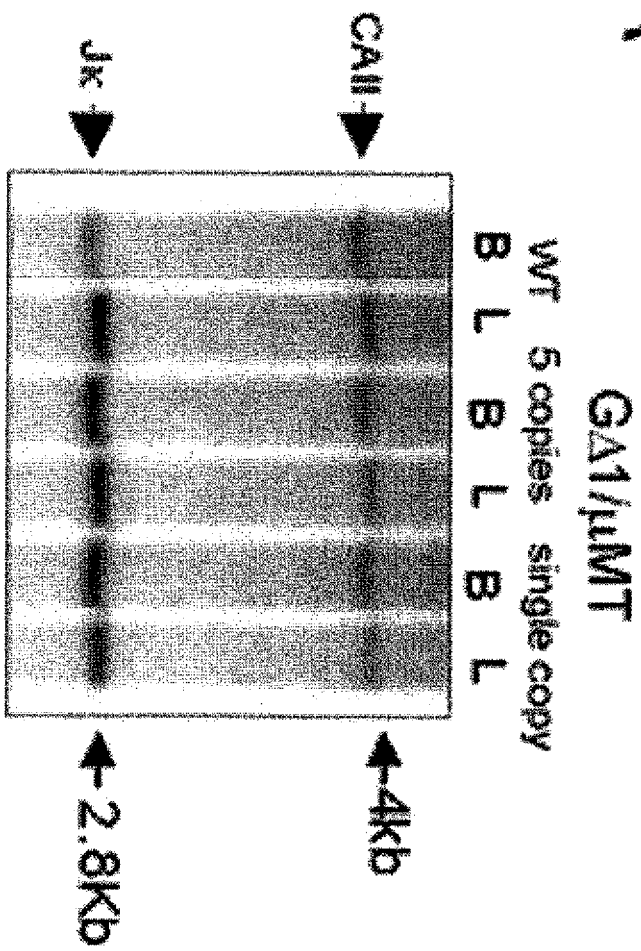


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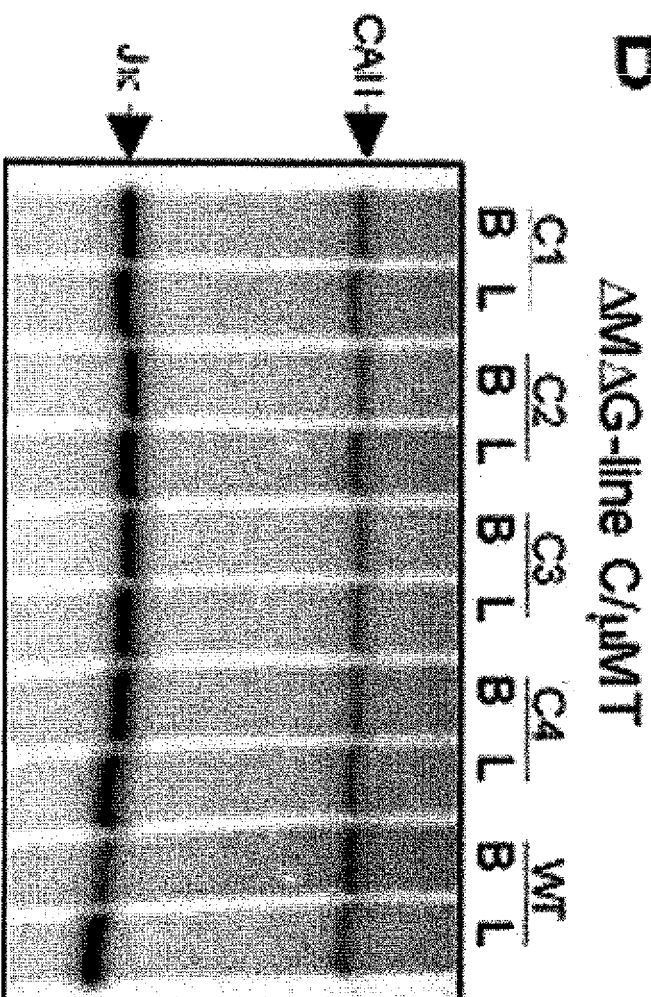
	CH1	IVS I
Human $\gamma$ 2	ACAAGACAGTTG	GTGAGAGG
Human $\gamma$ 3	ACAAGAGAGTTG	GTGAGAGG
Camel $\gamma$ 2a (Nguyen <i>et al.</i> )	ACAAGAGTGTGG	ATAAGTAG
MGS or GS $\gamma$ 2	ACAAGACAGTTG	ATGAGAGG
MGS or GS $\gamma$ 3	ACAAGAGAGTTG	ATGAGAGG
patient (Zhao <i>et al.</i> )	ACAAGACAGTTG	GTGGGAGG



**A**



**B**



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(54) Title: PRODUCTION OF HUMANIZED ANTIBODIES IN TRANSGENIC ANIMALS

(57) Abstract: This invention relates to humanized antibodies and antibody preparations produced from transgenic non-human animals. The non-human animals are genetically engineered to contain one or more humanized immunoglobulin loci which are capable of undergoing gene rearrangement and gene conversion in the transgenic non-human animals to produce diversified humanized immunoglobulins. The present invention further relates to novel sequences, recombination vectors and transgenic vectors useful for making these transgenic animals. The humanized antibodies of the present invention have minimal immunogenicity to humans and are appropriate for use in the therapeutic treatment of human subjects.

## **Production of Humanized Antibodies In Transgenic Animals**

### **5 Field of the Invention**

This invention relates to humanized antibodies produced from transgenic non-human animals. The non-human animals are genetically engineered to contain one or more humanized immunoglobulin loci which are capable of undergoing gene rearrangement and gene conversion in the transgenic non-human animals to produce  
10 diversified humanized immunoglobulins. The present invention further relates to novel sequences, recombination vectors and transgenic vectors useful for making these transgenic animals. The humanized antibodies of the present invention have minimal immunogenicity to humans and are appropriate for use in the therapeutic treatment of human subjects.

15

### **Background of the Invention**

The therapy of infectious diseases caused by bacteria, fungi, virus and parasites is largely based on chemotherapy. However, the emergence of drug-resistant organisms requires the continuous development of new antibiotics. Therapies of patients with  
20 malignancies and cancer are also based on chemotherapy. However, many of these therapies are ineffective and the mortality of diseased patients is high. For both infectious diseases and cancer, improved and innovative therapies are needed. Therapy of steroid resistant rejection of transplanted organs requires the use of biological reagents (monoclonal or polyclonal antibody preparations) that reverse the ongoing  
25 alloimmune response in the transplant recipient. The major problem of antibody preparations obtained from animals is the intrinsic immunogenicity of non-human immunoglobulins in human patients. In order to reduce the immunogenicity of non-human antibodies, genetic engineering of individual antibody genes in animals has been proposed. In particular, it has been shown that by fusing animal variable (V) region exons  
30 with human constant (C) region exons, a chimeric antibody gene can be obtained. However, this approach may only eliminate the immunogenicity caused by the non-human

Fc region, while the remaining non-human Fab sequences may still be immunogenic. In another approach, human immunoglobulin genes for both, heavy and light chain immunoglobulins have been introduced into the genome of mice. While this genetic engineering approach resulted in the expression of human immunoglobulin polypeptides in genetically engineered mice, the level of human immunoglobulin expression is low. This may be due to species-specific regulatory elements in the immunoglobulin loci that are necessary for efficient expression of immunoglobulins. As demonstrated in transfected cell lines, regulatory elements present in human immunoglobulin genes may not function properly in non-human animals.

Several regulatory elements in immunoglobulin genes have been described. Of particular importance are enhancers downstream (3') of heavy chain constant regions and intronic enhancers in light chain genes. In addition, other, yet to be identified, control elements may be present in immunoglobulin genes. Studies in mice have shown that the membrane and cytoplasmic tail of the membrane form of immunoglobulin molecules play an important role in expression levels of human-mouse chimeric antibodies in the serum of mice homozygous for the human C $\gamma$ 1 gene. Therefore, for the expression of heterologous immunoglobulin genes in animals it is desirable to replace sequences that contain enhancer elements and exons encoding transmembrane (M1 exon) and cytoplasmic tail (M2 exon) with sequences that are normally found in the animal in similar positions.

The introduction of human immunoglobulin genes into the genome of mice resulted in expression of a diversified human antibody repertoire in genetically engineered mice. In both mice and humans, antibody diversity is generated by gene rearrangement. This process results in the generation of many different recombined V(D)J segments encoding a large number of antibody molecules with different antigen binding sites. However, in other animals, like rabbits, pigs, cows and birds, antibody diversity is generated by a substantially different mechanism called gene conversion. For example, it is well established that in rabbit and chicken, VDJ rearrangement is very limited (almost 90% of immunoglobulin is generated with the 3'proximal VH1 element) and antibody diversity is generated by gene conversion and hypermutation. In contrast, mouse and

human gene conversion occurs very rarely, if at all. Therefore, it is expected that in animals that diversify antibodies by gene conversion a genetic engineering approach based on gene rearrangement will result in animals with low antibody titers and limited antibody diversity. Thus, the genetic engineering of large animals for the production of non-immunogenic antibody preparations for human therapy requires alternative genetic engineering strategies.

### Relevant Literature

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Production of antibodies from transgenic animals is described in U.S. Patent No. 5,814,318, No. 5,545,807 and No. 5,570,429. Homologous recombination for chimeric mammalian hosts is exemplified in U.S. Patent No. 5,416,260. A method for  
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5

### Summary of the Invention

One embodiment of the present invention provides humanized antibodies (humanized immunoglobulins) having at least a portion of a human immunoglobulin polypeptide sequence.

10

The humanized antibodies of the present invention are made from transgenic non-human animals genetically engineered to contain one or more humanized Ig loci.

Preferably, the humanized antibodies of the present invention are prepared from transgenic non-human animals which generate antibody diversity primarily by gene conversion and hypermutation, e.g., rabbit, pigs, chicken, sheep, cow and horse. The antibodies can be made by immunizing transgenic animals with a desired antigen such as an infectious agent (e.g., bacteria or viruses) or parts or fragments thereof.

15

Such humanized antibodies have reduced immunogenicity to primates, especially humans, as compared to non-humanized antibodies prepared from non-human animals. Therefore, the humanized antibodies of the present invention are appropriate for use in the therapeutic treatment of human subjects.

20

Another embodiment of the present invention provides a preparation of humanized antibodies which can be monoclonal antibodies or polyclonal antibodies. Preferred antibody preparations of the present invention are polyclonal antibody preparations which, according to the present invention, have minimal immunogenicity to primates, especially humans.

25

A preferred preparation of polyclonal antibodies is composed of humanized immunoglobulin molecules having at least a heavy chain or light chain constant region polypeptide sequence encoded by a human constant region gene segment. More preferably, the variable domains of the heavy chains or light chains of the immunoglobulins molecules are also encoded by human gene segments.

30

In another embodiment, the present invention provides pharmaceutical compositions which include a preparation of humanized antibodies, and a pharmaceutically-acceptable carrier.

Another embodiment of the present invention provides novel sequences from the 5' and 3' flanking regions of the Ig gene segments of non-human animals, preferably, animals which rely primarily on gene conversion in generating the antibody diversity. In particular, the present invention provides novel nucleotide sequences downstream (3', 3-prime) of the genes coding for C $\lambda$  in chickens, C $\gamma$  and C $\kappa$  in rabbits, C $\gamma$ 1,2,3 in cows and C $\gamma$ 1,2 in sheep, as well as novel sequences 5' of rabbit C $\gamma$ .

In another embodiment, the present invention provides recombination vectors useful for replacing an Ig gene segment of a non-human animal with the corresponding human Ig gene segment. These vectors include a human Ig gene segment which is linked to flanking sequences at the 5' end and the 3' end, wherein the flanking sequences are homologous to the flanking sequences of the target animal Ig gene segment.

Preferred recombination vectors are those useful for the replacement of the animal's Ig constant region. For example, recombination vectors useful for replacing the rabbit heavy chain constant region genes are provided. A preferred vector contains from 5' to 3', a nucleotide sequence as set forth in SEQ ID NO: 12 or SEQ ID NO: 13, or a portion of SEQ ID NO: 12 or SEQ ID NO: 13, a human heavy chain constant region gene segment, a nucleotide sequence as set forth in SEQ ID NO: 10 or a portion of or SEQ ID NO: 10. Another preferred vector contains a nucleotide sequence as set forth in SEQ ID NO: 51, which sequence is characterized as having a human C $\gamma$ 1 gene linked to flanking sequences from the 5' and 3' flanking regions of a rabbit heavy chain constant region gene.

Recombination vectors are also provided useful for replacing the rabbit light chain constant region genes. A preferred vector contains a nucleotide sequence as set forth in SEQ ID NO: 53, which sequence is characterized as having a human C $\kappa$  linked to flanking sequences from the 5' and 3' flanking regions of the rabbit light chain C $\kappa$ 1 gene.

Other recombination vectors are provided which are useful for replacing the chicken light chain constant region genes. A preferred vector contains a nucleotide

sequence as set forth in SEQ ID NO: 57 which is characterized as having a human C $\lambda$ 2 linked to flanking sequences from the 5' and 3' flanking regions of the chicken light chain C $\lambda$  gene.

Other recombination vectors provided include those useful for replacing the animal's Ig V region elements. For example, a recombination vector useful for replacing a rabbit heavy chain V region element is provided and contains SEQ ID NO: 52. A recombination vector useful for replacing a rabbit light chain V region element is provided and contains SEQ ID NO: 54.

In still another embodiment, the present invention provides transgenic constructs or vectors containing at least one humanized Ig locus, i.e., an Ig locus from a non-human animal or a portion of an Ig locus from a non-human animal wherein the locus or the portion of a locus is genetically modified to contain at least one human Ig gene segment. Such humanized Ig locus has the capacity to undergo gene rearrangement and gene conversion in the non-human animal thereby producing a diversified repertoire of humanized immunoglobulins.

One humanized Ig locus provided by the invention is a humanized heavy chain locus which includes one or more V gene segments, one or more D gene segments, one or more J gene segments, and one or more constant region gene segments, wherein at least one gene segment is a human heavy chain gene segment. The gene segments in the humanized heavy chain locus are juxtaposed with respect to each other in an unrearranged, or partially or fully rearranged configuration. A preferred humanized heavy chain locus contains a human constant region gene segment, preferably, C $\alpha$  or C $\gamma$ . A more preferred humanized locus contains multiple V gene segments and at least one human V gene segment, in addition to a human heavy chain constant region segment. The human V gene segment is placed downstream of the non-human V gene segments.

Another humanized Ig locus is a humanized light chain locus which includes one or more V gene segments, one or more J gene segments, and one or more constant region gene segments, wherein at least one gene segment is a human light chain gene segment. The gene segments in the humanized light chain locus are juxtaposed with respect to each other in an unrearranged or rearranged configuration. A preferred

humanized light chain locus contains a human constant region gene segment, preferably, C $\lambda$  or C $\kappa$ . More preferably, the humanized light chain locus further contains multiple V gene segments and at least one human V gene segment. The human V gene segment is placed downstream of the non-human V gene segments. Even more preferably, the  
5 humanized light chain locus includes a rearranged human VJ segment, placed downstream of a number of (e.g., 10-100) VL gene segments of either non-human or human origin.

Another embodiment of the present invention is directed to methods of making a transgenic vector containing a humanized Ig locus by isolating an Ig locus or a portion of an Ig locus from a non-human animal, and integrating the desired human Ig gene  
10 segment(s) into the isolated animal Ig locus or the isolated portion of an Ig locus. The human Ig gene segment(s) are integrated into the isolated animal Ig locus or the isolated portion of an Ig locus by ligation or homologous recombination in such a way as to retain the capacity of the locus for undergoing effective gene rearrangement and gene conversion in the non-human animal. Integration of a human Ig gene segment by  
15 homologous recombination can be accomplished by using the recombination vectors of the present invention.

In another embodiment, the present invention provides methods of making transgenic animals capable of producing humanized antibodies. The transgenic animals can be made by introducing a transgenic vector containing a humanized Ig locus, or a  
20 recombination vector containing a human Ig gene segment, into a recipient cell or cells of an animal, and deriving an animal from the genetically modified recipient cell or cells.

Transgenic animals containing one or more humanized Ig loci, and cells derived from such transgenic animals (such as B cells from an immunized transgenic animal) are also provided. The transgenic animals of the present invention are capable of  
25 gene rearranging and gene converting the transgenic humanized Ig loci to produce a diversified repertoire of humanized immunoglobulin molecules.

### Brief Description of the Drawings

**Figure 1.** Cow Cy 3' flanking sequences. Primers are shown in shaded boxes.  
30 The 5' primer is in CH3, and the 3' primer is in M1. The sequences of clone 11, clone 3,

and clone 5 are set forth in SEQ ID NO: 3, SEQ ID NO: 4 and SEQ ID NO: 5, respectively.

**Figure 2.** Sheep Cy 3' flanking sequences. Primers are shown in shaded boxes. The 5' primer is in CH3, and the 3' primer is in M2. The sequences of clone 11 and clone 1 are set forth in SEQ ID NO: 8 and SEQ ID NO: 9, respectively.

**Figure 3.** A novel 3' flanking sequence (SEQ ID NO: 10) of the rabbit Cgamma gene.

**Figure 4.** A novel nucleotide sequence (SEQ ID NO: 11) 3' of the rabbit Ckappa 1 gene.

**Figure 5.** Novel nucleotide sequences (SEQ ID NO: 12 and SEQ ID NO: 13) 5' of the rabbit Cgamma gene. The sequences between SEQ ID NO: 12 and SEQ ID NO: 13 (a gap of about 1000 nt) remain to be determined.

**Figure 6.** Comparison of human, mouse, rabbit, sheep, cow and camel sequences for the M1 and M2 regions 3' of the Cgamma gene.

**Figure 7a.** DNA construct for the replacement of rabbit Ck with human Ck. A 0.5 kb fragment containing a DNA sequence encoding human Ck is flanked by sequences from the rabbit Ck1 gene. The upstream sequence (5'Ck) is 2.8 kb, the downstream sequence (3'Ck) is 2.6 kb. The vector also contains a lox-neo cassette for positive selection and a Hsv-Tk cassette for negative selection.

**Figure 7b.** DNA construct for the replacement of rabbit Cy with human Cy1. A 1.8 kb fragment containing a DNA sequence encoding human Cy1 is flanked by sequences from the rabbit Cy gene. The upstream sequence (5'Cy) is 1.9 kb, the downstream sequence (3'Cy) is 3.1 kb. The vector also contains a lox-neo cassette for positive selection and a Hsv-Tk cassette for negative selection. The figure is not up to scale.

**Figure 8.** DNA fragment (SEQ ID NO: 51) containing a human immunoglobulin heavy chain Cy1 gene segment flanked by 50 nucleotides derived from the flanking regions of rabbit Cy gene. Flanking sequences derived from the flanking regions of rabbit Cy gene are underlined.

**Figure 9.** DNA fragment (SEQ ID NO: 52) containing a V gene segment with more than 80% sequence identity with rabbit V elements and encoding a human V element polypeptide sequence. Flanking sequences derived from the flanking regions of rabbit VH1 and J genes are underlined.

5       **Figure 10.** DNA fragment (SEQ ID NO: 53) containing a human immunoglobulin heavy chain C $\kappa$  gene segment flanked by 50 nucleotides derived from the rabbit light chain immunoglobulin Kappa1 gene. Flanking sequences derived from the flanking regions of rabbit C $\kappa$  gene are underlined.

10       **Figure 11.** DNA fragment (SEQ ID NO: 54) containing a V gene segment with more than 80% sequence identity with rabbit V elements and encoding a human V element polypeptide sequence. Flanking sequences derived from the flanking regions of rabbit immunoglobulin V and J genes are underlined.

15       **Figure 12.** DNA fragment (SEQ ID NO: 57) containing a gene encoding human immunoglobulin light chain constant region Clambda2 flanked by 50 nucleotides (underlined) derived from the flanking sequences of chicken Clambda gene.

20       **Figure 13.** Modification of the chicken light chain locus using the ET system. A chicken genomic BAC clone with the full-length light chain locus was modified by homologous recombination. In a first step C $\lambda$  was deleted by insertion of a selection cassette which was in a second homologous recombination step exchanged against the human C $\lambda$  gene.

25       **Figure 14.** DNA fragment (SEQ ID NO: 58) containing a VJ gene segment with 80% sequence identity with chicken V gene segments and encoding a human VJ immunoglobulin polypeptide. Flanking sequences derived from the flanking regions of chicken immunoglobulin V and J genes are underlined.

**Figure 15.** Modified chicken light chain locus.

#### Detailed Description of the Invention

One embodiment of the present invention provides humanized immunoglobulins (antibodies).

By "a humanized antibody" or "a humanized immunoglobulin" is meant an immunoglobulin molecule having at least a portion of a human immunoglobulin polypeptide sequence (or a polypeptide sequence encoded by a human Ig gene segment). The humanized immunoglobulin molecules of the present invention can be isolated from a transgenic non-human animal engineered to produce humanized immunoglobulin molecules. Such humanized immunoglobulin molecules are less immunogenic to primates, especially humans, relative to non-humanized immunoglobulin molecules prepared from the animal or prepared from cells derived from the animal.

The term "non-human animals" as used herein includes, but is not limited to, rabbits, pigs, birds (e.g., chickens, turkeys, ducks, geese and the like), sheep, goats, cows and horses. Preferred non-human animals are those animals which rely primarily on gene conversion and/or somatic hypermutation to generate antibody diversity, e.g., rabbit, pigs, birds (e.g., chicken, turkey, duck, goose and the like), sheep, goat, and cow. Particularly preferred non-human animals are rabbit and chicken.

In animals such as human and mouse, there are multiple copies of V, D and J gene segments on the heavy chain locus, and multiple copies of V and J gene segments on a light chain locus. Antibody diversity in these animals is generated primarily by gene rearrangement, i.e., different combinations of gene segments to form rearranged heavy chain variable region and light chain variable region. In other animals (e.g., rabbit, chicken, sheep, goat, and cow), however, gene rearrangement does not play a significant role in the generation of antibody diversity. For example, in rabbit, only a very limited number of the V gene segments, most often the V gene segments at the 3' end of the V-region, are used in gene rearrangement to form a contiguous VDJ segment. In chicken, only one V gene segment (the one adjacent to the D region, or "the 3' proximal V gene segment"), one D segment and one J segment are used in the heavy chain rearrangement; and only one V gene segment (the 3' proximal V segment) and one J segment are used in the light chain rearrangement. Thus, in these animals, there is little diversity among initially rearranged variable region sequences resulting from junctional diversification. Further diversification of the rearranged Ig genes is achieved by gene conversion, a

process in which short sequences derived from the upstream V gene segments replace short sequences within the V gene segment in the rearranged Ig gene.

The term "Ig gene segment" as used herein refers to segments of DNA encoding various portions of an Ig molecule, which are present in the germline of animals and humans, and which are brought together in B cells to form rearranged Ig genes. Thus, Ig gene segments as used herein include V gene segments, D gene segments, J gene segments and C region gene segments.

The term "human Ig gene segment" as used herein includes both naturally occurring sequences of a human Ig gene segment, degenerate forms of naturally occurring sequences of a human Ig gene segment, as well as synthetic sequences that encode a polypeptide sequence substantially identical to the polypeptide encoded by a naturally occurring sequence of a human Ig gene segment. By "substantially" is meant that the degree of amino acid sequence identity is at least about 85%-95%.

A preferred humanized immunoglobulin molecule of the present invention contains at least a portion of a human heavy or light chain constant region polypeptide sequence. A more preferred immunoglobulin molecule contains at least a portion of a human heavy or light chain constant region polypeptide sequence, and at least a portion of a human variable domain polypeptide sequence.

In another embodiment of the present invention, a preparation of humanized antibodies is provided.

By "a preparation of humanized antibodies" or "a humanized antibody preparation" is meant an isolated antibody product or a purified antibody product prepared from a transgenic non-human animal (e.g., serum, milk, or egg yolk of the animal) or from cells derived from a transgenic non-human animal (e.g., a B-cell or a hybridoma cell).

A humanized antibody preparation can be a preparation of polyclonal antibodies, which includes a repertoire of humanized immunoglobulin molecules. A humanized antibody preparation can also be a preparation of a monoclonal antibody.

Although the immunogenicity to humans of a humanized monoclonal antibody preparation is also reduced as compared to a non-humanized monoclonal antibody preparation, humanized polyclonal antibody preparations are preferred embodiments of

the present invention. It has been recognized that humanized monoclonal antibodies still invoke some degree of an immune response (an anti-idiotypic response) in primates (e.g., humans) when administered repeatedly in large quantities because of the unique and novel idiotype of the monoclonal antibody. The present inventors have uniquely recognized that  
5 the overall immunogenicity of polyclonal antibodies is less dependent on an anti-idiotypic response. For example, polyclonal antibodies made from non-human animals with only the constant region elements humanized (e.g., polyclonal antibodies having constant regions encoded by human gene segments, and having variable domains encoded by the endogenous genes of the non-human animal), are substantially non-immunogenic to  
10 primates.

Without intending to be bound to any theory, the present inventors have proposed that the reduced immunogenicity of such a humanized polyclonal antibody preparation is due to the fact that the preparation contains a very large number of different antibodies with many different idiotypes which are to a large extent defined by novel  
15 amino acid sequences in the complementarity determining regions (CDR) of the heavy and light chain. Therefore, upon administration of such preparation into a primate such as a human, the administered amount of each individual immunoglobulin molecule in the preparation may be too low to solicit immune response against each immunoglobulin molecule. Thus, the humanized polyclonal antibody preparation which has many different  
20 idiotypes and variable regions has minimal immunogenicity to a recipient, even if the antibodies in the polyclonal antibody preparation are all directed to the same antigen. To further reduce any potential residual immunogenicity, a humanized polyclonal antibody preparation may be prepared which is composed of immunoglobulin molecules having both the variable domains and the constant regions encoded by human Ig gene segments.

25 In a preferred embodiment, the present invention provides an antibody preparation which includes humanized immunoglobulin molecules having at least a portion of a human heavy or light chain constant region polypeptide sequence. More preferably, the humanized immunoglobulins in the antibody preparation of the present invention further contain at least a portion of a human variable domain polypeptide

sequence, in addition to at least a portion of a human constant region polypeptide sequence.

Preferred humanized antibody preparations of the present invention are composed of humanized antibodies made from transgenic non-human animals whose  
5 antibody diversity is generated primarily by gene conversion, such as rabbit, birds (e.g., chicken, turkey, duck, goose and the like), sheep, goat, and cow; preferably, rabbit and chicken.

Once a transgenic non-human animal capable of producing diversified humanized immunoglobulin molecules is made (as further set forth below), humanized  
10 immunoglobulins and humanized antibody preparations against an antigen can be readily obtained by immunizing the animal with the antigen. A variety of antigens can be used to immunize a transgenic host animal. Such antigens include, microorganism, e.g. viruses and unicellular organisms (such as bacteria and fungi), alive, attenuated or dead, fragments of the microorganisms, or antigenic molecules isolated from the  
15 microorganisms.

Preferred bacterial antigens for use in immunizing an animal include purified antigens from *Staphylococcus aureus* such as capsular polysaccharides type 5 and 8, recombinant versions of virulence factors such as alpha-toxin, adhesin binding proteins, collagen binding proteins, and fibronectin binding proteins. Preferred bacterial antigens  
20 also include an attenuated version of *S. aureus*, *Pseudomonas aeruginosa*, enterococcus, enterobacter, and *Klebsiella pneumoniae*, or culture supernatant from these bacteria cells. Other bacterial antigens which can be used in immunization include purified lipopolysaccharide (LPS), capsular antigens, capsular polysaccharides and/or recombinant versions of the outer membrane proteins, fibronectin binding proteins, endotoxin, and  
25 exotoxin from *Pseudomonas aeruginosa*, enterococcus, enterobacter, and *Klebsiella pneumoniae*.

Preferred antigens for the generation of antibodies against fungi include attenuated version of fungi or outer membrane proteins thereof, which fungi include, but are not limited to, *Candida albicans*, *Candida parapsilosis*, *Candida tropicalis*, and  
30 *Cryptococcus neoformans*.

Preferred antigens for use in immunization in order to generate antibodies against viruses include the envelop proteins and attenuated versions of viruses which include, but are not limited to respiratory syncytial virus (RSV) (particularly the F-Protein), Hepatitis C virus (HCV), Hepatitis B virus (HBV), cytomegalovirus (CMV), EBV, and  
5 HSV.

Therapeutic antibodies can be generated for the treatment of cancer by immunizing transgenic animals with isolated tumor cells or tumor cell lines; tumor-associated antigens which include, but are not limited to, Her-2-neu antigen (antibodies against which are useful for the treatment of breast cancer); CD20, CD22 and CD53  
10 antigens (antibodies against which are useful for the treatment of B cell lymphomas), (3) prostate specific membrane antigen (PMSA) (antibodies against which are useful for the treatment of prostate cancer), and 17-1A molecule (antibodies against which are useful for the treatment of colon cancer).

The antigens can be administered to a transgenic host animal in any convenient  
15 manner, with or without an adjuvant, and can be administered in accordance with a predetermined schedule.

After immunization, serum or milk from the immunized transgenic animals can be fractionated for the purification of pharmaceutical grade polyclonal antibodies specific for the antigen. In the case of transgenic birds, antibodies can also be made by  
20 fractionating egg yolks. A concentrated, purified immunoglobulin fraction may be obtained by chromatography (affinity, ionic exchange, gel filtration, etc.), selective precipitation with salts such as ammonium sulfate, organic solvents such as ethanol, or polymers such as polyethyleneglycol.

For making a monoclonal antibody, spleen cells are isolated from the  
25 immunized transgenic animal and used either in cell fusion with transformed cell lines for the production of hybridomas, or cDNAs encoding antibodies are cloned by standard molecular biology techniques and expressed in transfected cells. The procedures for making monoclonal antibodies are well established in the art. See, e.g., European Patent Application 0 583 980 A1 ("Method For Generating Monoclonal Antibodies From  
30 Rabbits"), U.S. Patent No. 4,977,081 ("Stable Rabbit-Mouse Hybridomas And Secretion

Products Thereof”), WO 97/16537 (“Stable Chicken B-cell Line And Method of Use Thereof”), and EP 0 491 057 B1 (“Hybridoma Which Produces Avian Specific Immunoglobulin G”), the disclosures of which are incorporated herein by reference. In vitro production of monoclonal antibodies from cloned cDNA molecules has been  
5 described by Andris-Widhopf et al., “Methods for the generation of chicken monoclonal antibody fragments by phage display”, *J Immunol Methods* 242:159 (2000), and by Burton, D. R., “Phage display”, *Immunotechnology* 1:87 (1995), the disclosures of which are incorporated herein by reference.

In a further embodiment of the present invention, purified monoclonal or  
10 polyclonal antibodies are admixed with an appropriate pharmaceutical carrier suitable for administration in primates especially humans, to provide pharmaceutical compositions. Pharmaceutically acceptable carriers which can be employed in the present pharmaceutical compositions can be any and all solvents, dispersion media, isotonic agents and the like. Except insofar as any conventional media, agent, diluent or carrier is  
15 detrimental to the recipient or to the therapeutic effectiveness of the antibodies contained therein, its use in the pharmaceutical compositions of the present invention is appropriate. The carrier can be liquid, semi-solid, e.g. pastes, or solid carriers. Examples of carriers include oils, water, saline solutions, alcohol, sugar, gel, lipids, liposomes, resins, porous matrices, binders, fillers, coatings, preservatives and the like, or combinations thereof

20 The present invention is further directed to novel nucleotide sequences and vectors, as well as the use of the sequences and vectors in making a transgenic non-human animal which produces humanized immunoglobulins.

In general, the genetic engineering of a non-human animal involves the integration of one or more human Ig gene segments into the animal's genome to create  
25 one or more humanized Ig loci. It should be recognized that, depending upon the approach used in the genetic modification, a human Ig gene segment can be integrated at the endogenous Ig locus of the animal (as a result of targeted insertion, for example), or at a different locus of the animal. In other words, a humanized Ig locus can reside at the chromosomal location where the endogenous Ig locus of the animal ordinarily resides, or  
30 at a chromosomal location other than where the endogenous Ig locus of the animal

ordinarily resides. Regardless of the chromosomal location, a humanized Ig locus of the present invention has the capacity to undergo gene rearrangement and gene conversion in the non-human animal thereby producing a diversified repertoire of humanized immunoglobulin molecules. An Ig locus having the capacity to undergo gene  
5 rearrangement and gene conversion is also referred to herein as a "functional" Ig locus, and the antibodies with a diversity generated by a functional Ig locus are also referred to herein as "functional" antibodies or a "functional" repertoire of antibodies.

In one embodiment, the present invention provides novel sequences useful for creating a humanized Ig locus and making transgenic animals capable of producing  
10 humanized immunoglobulin molecules. In particular, the present invention provides sequences from the 5' and 3' flanking regions of the Ig gene segments of non-human animals, preferably, animals which rely primarily on gene conversion in generating antibody diversity (e.g., rabbit, pigs, sheep, goat, cow, birds such as chicken, turkey, duck, goose, and the like).

15 The 5' and 3' flanking regions of the genes coding for the constant region are particularly important as these sequences contain untranslated regulatory elements (e.g., enhancers) critical for high Ig expression in the serum. The 3' flanking region of the genes coding for the constant region of the heavy chain also contain exons coding for the membranous and cytoplasmic tail of the membrane form of immunoglobulin (Volgina et  
20 al. *J Immunol* 165:6400, 2000). It has been previously established that the membrane and cytoplasmic tail of the membrane form of antibodies are critical in achieving a high level of expression of the antibodies in mice sera (Zou et al., *Science* 262:1271, 1993). Thus, the identification of the flanking sequences permits the replacement of exons and intervening introns of the Cy gene with the human equivalent, and the maintenance of the  
25 endogenous exons encoding the transmembrane and cytoplasmic tail regions as well as the endogenous non-coding enhancer sequences.

In one embodiment, the present invention provides 3' flanking sequences of heavy chain constant regions of non-human animals. More particularly, nucleotide sequences downstream (3', 3-prime) of the genes coding for rabbit Cy, cow Cy1,2,3, and  
30 sheep Cy1,2 are provided. Especially preferred nucleotide sequences include SEQ ID NO:

10 (3' of rabbit  $C\gamma$ ), SEQ ID NOS: 3-5 (3' of cow  $C\gamma_{1,2,3}$ ), and SEQ ID NOS: 8-9 (3' of sheep  $C\gamma_{1,2}$ ).

In another embodiment, the present invention provides 3' flanking sequences of light chain constant regions of non-human animals. More particularly, the present invention provides nucleotide sequences downstream (3', 3-prime) of the genes coding for  $C\kappa$  in rabbits. Especially preferred nucleotide sequences include SEQ ID NO: 11 (3' of rabbit  $C\kappa$ ).

In still another embodiment, the present invention provides 5' flanking sequences of heavy chain constant regions of non-human animals. More particularly, nucleotide sequences upstream (5', 5-prime) of the rabbit  $C\gamma$  gene are provided. Especially preferred sequences include SEQ ID NO: 12 and SEQ ID NO: 13.

Another embodiment of the present invention provides 5' flanking sequences of light chain constant regions of non-human animals.

Portions of the above novel flanking sequences are provided by the present invention. By "a portion" is meant a fragment of a flanking nucleotide sequence capable of mediating homologous recombination between the human Ig gene segment and the target animal Ig gene segment. Generally, a portion is at least about 200 base pairs, preferably, at least about 400 base pairs, for recombination in animal cells such as ES cells or fibroblasts, and at least about 40 base pairs, preferably at least about 50 base pairs, for recombination in *E. coli*. Examples of portions of the above novel flanking sequences include SEQ ID NOS: 59-60, 61-62, 63-64, 65-66, 67-68 and 69-70 (represented by the underlined sequences in Figures 8-12 and 14, respectively).

In a further aspect, the present invention provides vectors useful for the replacement of an Ig gene segment of a non-human animal with the corresponding human Ig gene segment. These vectors, also referred to herein as "recombination vectors", include a human Ig gene segment which is linked to flanking sequences at the 5' end and the 3' end, wherein the flanking sequences have a degree of homology with the flanking sequences of the target animal Ig gene segment sufficient to mediate homologous recombination between the human gene and the animal gene segments. Generally, at least about 200 bases should be identical between the flanking regions in a recombination

vector and the flanking regions of the target gene to achieve efficient homologous recombination in animal cells such as ES cells and fibroblasts; and at least about 40 bases should be identical to achieve efficient homologous recombination in *E. coli*.

Recombination vectors useful for replacing the animal's immunoglobulin heavy chain constant region genes are provided, which contain from 5' to 3', a nucleotide sequence homologous to the 5' flanking region of the target animal heavy chain constant region gene, a human heavy chain constant region gene (e.g., human C $\gamma$ 1), and a nucleotide sequence homologous to the 3' flanking region of the target animal heavy chain constant region gene.

Preferred recombination vectors are provided for the replacement of the rabbit heavy chain constant region genes. One such vector contains from 5' to 3', a nucleotide sequence as set forth in SEQ ID NO: 12 or SEQ ID NO: 13 or a portion thereof, a human heavy chain constant region gene segment, a nucleotide sequence as set forth in SEQ ID NO: 10 or a portion of or SEQ ID NO: 10. Another such vector contains SEQ ID NO: 51 (Figure 8) which is characterized as having a human C $\gamma$ 1 gene linked to flanking sequences from the 5' and 3' flanking regions of a rabbit heavy chain constant region gene.

Recombination vectors are also provided which are useful for replacing the animal's immunoglobulin light chain constant region genes. Such vectors contain from 5' to 3', a nucleotide sequence homologous to the 5' flanking region of the target light chain constant region gene, a human light chain constant region gene (e.g., human C $\kappa$  or C $\lambda$ ), and a nucleotide sequence homologous to the 3' flanking region of the target light chain constant region gene.

Preferred vectors include those useful for replacing the rabbit light chain constant region genes. A preferred vector contains a nucleotide sequence as set forth in SEQ ID NO: 53, which sequence is characterized as having a human C $\kappa$  linked to flanking sequences from the 5' and 3' flanking regions of the rabbit light chain C $\kappa$ 1 gene.

Other recombination vectors provided include those useful for replacing the animal's Ig V region elements. For example, a recombination vector useful for replacing a rabbit heavy chain V region element is provided and contains SEQ ID NO: 52. A

recombination vector useful for replacing a rabbit light chain V region element is provided and contains SEQ ID NO: 54.

The recombination vectors of the present invention can include additional sequences that facilitate the selection of cells which have undergone a successful  
5 recombination event. For example, marker genes coding for resistance to neomycin, bleomycin, puromycin and the like can be included in the recombination vectors to facilitate the selection of cells which have undergone a successful recombination event.

In a further aspect of the present invention, transgenic constructs or vectors carrying one or more humanized Ig loci are provided.

10 In one embodiment, the present invention provides transgenic constructs containing a humanized Ig heavy chain locus which includes one or more V gene segments, one or more D gene segments, one or more J gene segments, and one or more constant region gene segments, wherein at least one gene segment is a human heavy chain gene segment. The gene segments in such humanized heavy chain locus are juxtaposed  
15 wit respect to each other in an unrearranged configuration (or "the germline configuration"), or in a partially or fully rearranged configuration. The humanized heavy chain locus has the capacity to undergo gene rearrangement (if the gene segments are not fully rearranged) and gene conversion in the non-human animal thereby producing a diversified repertoire of heavy chains having human polypeptide sequences, or  
20 "humanized heavy chains".

In a preferred embodiment, the humanized heavy chain locus contains at least one C-region gene segment that is a human constant region gene segment, preferably, C $\alpha$  or C $\gamma$  (including any of the C $\gamma$  subclasses 1, 2, 3 and 4).

In another more preferred embodiment, the humanized heavy chain locus of  
25 the transgene contains a humanized V-region and a humanized C-region, i.e., a V-region having at least one human VH gene segment and a C-region having at least one human C gene segment (e.g., human C $\alpha$  or C $\gamma$ ).

Preferably, the humanized V-region includes at least about 10-100 heavy chain V (or "VH") gene segments, at least one of which is a human VH gene segment. In  
30 accordance with the present invention, the human VH gene segment included in the

transgene shares at least about 75% to about 85% homology to the VH gene segments of the host animal, particularly those animal VH gene segments included in the upstream region of the transgene. As described above, a human VH segment encompasses naturally occurring sequences of a human VH gene segment, degenerate forms of naturally occurring sequences of a human VH gene segment, as well as synthetic sequences that  
5 encode a polypeptide sequence substantially (i.e., at least about 85%-95%) identical to a human heavy chain V domain polypeptide.

Preferably, the human VH gene segment(s) is placed downstream of the non-human VH segments in the transgene locus. Preferably, the non-human VH gene  
10 segments in the transgene are the VH gene segments from the 3' VH-region in the Ig locus of the host animal, including the 3' proximal VH1.

In another embodiment, the present invention provides transgenic constructs containing a humanized light chain locus capable of undergoing gene rearrangement and gene conversion in the host animal thereby producing a diversified repertoire of light  
15 chains having human polypeptide sequences, or "humanized light chains".

The humanized light locus includes one or more V gene segments, one or more J gene segments, and one or more constant region gene segments, wherein at least one gene segment is a human light chain gene segment. The gene segments in the humanized light chain locus are juxtaposed in an unrearranged configuration (or "the germline  
20 configuration"), or fully rearranged configuration.

In a preferred embodiment, the humanized light chain locus contains at least one C-region gene segment that is a human constant region gene segment, preferably, C $\lambda$  or C $\kappa$ .

In another preferred embodiment, the humanized light chain locus of the  
25 transgene contains a humanized V-region and a humanized C-region, e.g., a V-region having at least one human VL gene and/or at least one rearranged human VJ segment, and a C-region having at least one human C gene segment (e.g., human C $\lambda$  or C $\kappa$ ).

Preferably, the humanized V-region includes at least about 10-100 light chain V (or "VL") gene segments, at least one of which is a human VL gene segment. The  
30 human VL gene segment included in the transgene shares at least about 75% to about 85%

homology to the VL gene segments of the host animal, particularly those animal VL gene segments included in the upstream region of the transgene. Consistently, a human VL segment encompasses naturally occurring sequences of a human VL gene segment, degenerate forms of naturally occurring sequences of a human VL gene segment, as well  
5 as synthetic sequences that encode a polypeptide sequence substantially (i.e., at least about 85%-95%) identical to a human light chain V domain polypeptide.

Preferably, the human VL gene segment(s) is placed downstream of the non-human VL segments in the transgene locus. The non-human VL gene segments in the transgene construct are selected from the VL gene segments in the 3' VL-region in the  
10 light chain locus of the host animal, including the 3' proximal VL1.

In still another preferred embodiment, the humanized light chain locus includes a rearranged human VJ segment, placed downstream of a number of (e.g., 10-100) VL gene segments of either non-human or human origin.

Another aspect of the present invention is directed to methods of making a  
15 transgenic vector containing a humanized Ig locus. Such methods involve isolating an Ig locus or a portion thereof from a non-human animal, and inserting the desired human Ig gene segment(s) into the isolated animal Ig locus or the isolated portion of an animal Ig locus. The human Ig gene segment(s) are inserted into the isolated animal Ig locus or a portion thereof by ligation or homologous recombination in such a way as to retain the  
20 capacity of the locus of undergoing effective gene rearrangement and gene conversion in the non-human animal.

Preferably, DNA fragments containing an Ig locus to be humanized are isolated from animals which generate antibody diversity by gene conversion, e.g., rabbit and chicken. Such large DNA fragments can be isolated by screening a library of  
25 plasmids, cosmids, YACs or BACs, and the like, prepared from the genomic DNA of the non-human animal. An entire animal C-region can be contained in one plasmid or cosmid clone which is subsequently subjected to humanization. YAC clones can carry DNA fragments of up to 2 megabases, thus an entire animal heavy chain locus or a large portion thereof can be isolated in one YAC clone, or reconstructed to be contained in one YAC  
30 clone. BAC clones are capable of carrying DNA fragments of smaller sizes (about 150-

250 kb). However, multiple BAC clones containing overlapping fragments of an Ig locus can be separately humanized and subsequently injected together into an animal recipient cell, wherein the overlapping fragments recombine in the recipient animal cell to generate a continuous Ig locus.

5 Human Ig gene segments can be integrated into the Ig locus on a vector (e.g., a BAC clone) by a variety of methods, including ligation of DNA fragments, or insertion of DNA fragments by homologous recombination. Integration of the human Ig gene segments is done in such a way that the human Ig gene segment is operably linked to the host animal sequence in the transgene to produce a functional humanized Ig locus, i.e., an  
10 Ig locus capable of gene rearrangement and gene conversion which lead to the production of a diversified repertoire of humanized antibodies.

Preferably, human Ig gene segments are integrated into the Ig locus by homologous recombination. Homologous recombination can be performed in bacteria, yeast and other cells with a high frequency of homologous recombination events. For  
15 example, a yeast cell is transformed with a YAC containing an animal's Ig locus or a large portion thereof. Subsequently, such yeast cell is further transformed with a recombination vector as described hereinabove, which carries a human Ig gene segment linked to a 5' flanking sequence and a 3' flanking sequence. The 5' and the 3' flanking sequences in the recombination vector are homologous to those flanking sequences of the animal Ig gene  
20 segment on the YAC. As a result of a homologous recombination, the animal Ig gene segment on the YAC is replaced with the human Ig gene segment. Alternatively, a bacterial cell such as *E. coli* is transformed with a BAC containing an animal's Ig locus or a large portion thereof. Such bacterial cell is further transformed with a recombination vector which carries a human Ig gene segment linked to a 5' flanking sequence and a 3'  
25 flanking sequence. The 5' and the 3' flanking sequences in the recombination vector mediate homologous recombination and exchange between the human Ig gene segment on the recombination vector and the animal Ig gene segment on the BAC. Humanized YACs and BACs can be readily isolated from the cells and used in making transgenic animals.

In a further aspect of the present invention, methods of making transgenic  
30 animals capable of producing humanized immunoglobulins are provided.

According to the present invention, a transgenic animal capable of making humanized immunoglobulins are made by introducing into a recipient cell or cells of an animal one or more of the transgenic vectors described herein above which carry a humanized Ig locus, and deriving an animal from the genetically modified recipient cell or cells.

Preferably, the recipient cells are from non-human animals which generate antibody diversity by gene conversion and hypermutation, e.g., bird (such as chicken), rabbit, cows and the like. In such animals, the 3'proximal V gene segment is preferentially used for the production of immunoglobulins. Integration of a human V gene segment into the Ig locus on the transgene vector, either by replacing the 3'proximal V gene segment of the animal or by being placed in close proximity of the 3'proximal V gene segment, results in expression of human V region polypeptide sequences in the majority of immunoglobulins. Alternatively, a rearranged human V(D)J segment may be inserted into the J locus of the immunoglobulin locus on the transgene vector.

The transgenic vectors containing a humanized Ig locus is introduced into the recipient cell or cells and then integrated into the genome of the recipient cell or cells by random integration or by targeted integration.

For random integration, a transgenic vector containing a humanized Ig locus can be introduced into an animal recipient cell by standard transgenic technology. For example, a transgenic vector can be directly injected into the pronucleus of a fertilized oocyte. A transgenic vector can also be introduced by co-incubation of sperm with the transgenic vector before fertilization of the oocyte. Transgenic animals can be developed from fertilized oocytes. Another way to introduce a transgenic vector is by transfecting embryonic stem cells and subsequently injecting the genetically modified embryonic stem cells into developing embryos. Alternatively, a transgenic vector (naked or in combination with facilitating reagents) can be directly injected into a developing embryo. Ultimately, chimeric transgenic animals are produced from the embryos which contain the humanized Ig transgene integrated in the genome of at least some somatic cells of the transgenic animal.

In a preferred embodiment, a transgene containing a humanized Ig locus is randomly integrated into the genome of recipient cells (such as fertilized oocyte or developing embryos) derived from animal strains with an impaired expression of endogenous immunoglobulin genes. The use of such animal strains permits preferential  
5 expression of immunoglobulin molecules from the humanized transgenic Ig locus. Examples for such animals include the Alicia and Basilea rabbit strains, as well as Agammaglobulinemic chicken strain. Alternatively, transgenic animals with humanized immunoglobulin transgenes or loci can be mated with animal strains with impaired expression of endogenous immunoglobulins. Offspring homozygous for an impaired  
10 endogenous Ig locus and a humanized transgenic Ig locus can be obtained.

For targeted integration, a transgenic vector can be introduced into appropriate animal recipient cells such as embryonic stem cells or already differentiated somatic cells. Afterwards, cells in which the transgene has integrated into the animal genome and has replaced the corresponding endogenous Ig locus by homologous recombination can be  
15 selected by standard methods. The selected cells may then be fused with enucleated nuclear transfer unit cells, e.g. oocytes or embryonic stem cells, cells which are totipotent and capable of forming a functional neonate. Fusion is performed in accordance with conventional techniques which are well established. See, for example, Cibelli et al., Science (1998) 280:1256. Enucleation of oocytes and nuclear transfer can also be  
20 performed by microsurgery using injection pipettes. (See, for example, Wakayama et al., Nature (1998) 394:369.) The resulting egg cells are then cultivated in an appropriate medium, and transferred into synchronized recipients for generating transgenic animals. Alternatively, the selected genetically modified cells can be injected into developing embryos which are subsequently developed into chimeric animals.

25 Further to the present invention, a transgenic animal capable of producing humanized immunoglobulins can also be made by introducing into a recipient cell or cells, one or more of the recombination vectors described herein above, which carry a human Ig gene segment, linked to 5' and 3' flanking sequences that are homologous to the flanking sequences of the endogenous Ig gene segment, selecting cells in which the endogenous Ig

gene segment is replaced by the human Ig gene segment by homologous recombination, and deriving an animal from the selected genetically modified recipient cell or cells.

Similar to the target insertion of a transgenic vector, cells appropriate for use as recipient cells in this approach include embryonic stem cells or already differentiated  
5 somatic cells. A recombination vector carrying a human Ig gene segment can be introduced into such recipient cells by any feasible means, e.g., transfection. Afterwards, cells in which the human Ig gene segment has replaced the corresponding endogenous Ig gene segment by homologous recombination, can be selected by standard methods. These genetically modified cells can serve as nuclei donor cells in a nuclear transfer procedure  
10 for cloning a transgenic animal. Alternatively, the selected genetically modified embryonic stem cells can be injected into developing embryos which can be subsequently developed into chimeric animals.

Transgenic animals produced by any of the foregoing methods form another embodiment of the present invention. The transgenic animals have at least one, i.e., one  
15 or more, humanized Ig loci in the genome, from which a functional repertoire of humanized antibodies is produced.

In a preferred embodiment, the present invention provides transgenic rabbits having one or more humanized Ig loci in the genome. The transgenic rabbits of the present invention are capable of rearranging and gene converting the humanized Ig loci,  
20 and expressing a functional repertoire of humanized antibodies.

In another preferred embodiment, the present invention provides transgenic chickens having one or more humanized Ig loci in the genome. The transgenic chickens of the present invention are capable of rearranging and gene converting the humanized Ig loci, and expressing a functional repertoire of humanized antibodies.

25 Cells derived from the transgenic animals of the present invention, such as B cells or cell lines established from a transgenic animal immunized against an antigen, are also part of the present invention.

In a further aspect of the present invention, methods are provided for treating a disease in a primate, in particular, a human subject, by administering a purified humanized

antibody composition, preferably, a humanized polyclonal antibody composition, desirable for treating such disease.

The humanized polyclonal antibody compositions used for administration are generally characterized by containing a polyclonal antibody population, having  
5 immunoglobulin concentrations from 0.1 to 100 mg/ml, more usually from 1 to 10 mg/ml. The antibody composition may contain immunoglobulins of various isotypes. Alternatively, the antibody composition may contain antibodies of only one isotype, or a number of selected isotypes.

In most instances the antibody composition consists of unmodified  
10 immunoglobulins, i.e., humanized antibodies prepared from the animal without additional modification, e.g., by chemicals or enzymes. Alternatively, the immunoglobulin fraction may be subject to treatment such as enzymatic digestion (e.g. with pepsin, papain, plasmin, glycosidases, nucleases, etc.), heating, etc, and/or further fractionated.

The antibody compositions generally are administered into the vascular  
15 system, conveniently intravenously by injection or infusion via a catheter implanted into an appropriate vein. The antibody composition is administered at an appropriate rate, generally ranging from about 10 minutes to about 24 hours, more commonly from about 30 minutes to about 6 hours, in accordance with the rate at which the liquid can be accepted by the patient. Administration of the effective dosage may occur in a single  
20 infusion or in a series of infusions. Repeated infusions may be administered once a day, once a week once a month, or once every three months, depending on the half-life of the antibody preparation and the clinical indication. For applications on epithelial surfaces the antibody compositions are applied to the surface in need of treatment in an amount sufficient to provide the intended end result, and can be repeated as needed.

25 The antibody compositions can be used to bind and neutralize antigenic entities in human body tissues that cause disease or that elicit undesired or abnormal immune responses. An "antigenic entity" is herein defined to encompass any soluble or cell-surface bound molecules including proteins, as well as cells or infectious disease-causing organisms or agents that are at least capable of binding to an antibody and preferably are  
30 also capable of stimulating an immune response.

Administration of an antibody composition against an infectious agent as a monotherapy or in combination with chemotherapy results in elimination of infectious particles. A single administration of antibodies decreases the number of infectious particles generally 10 to 100 fold, more commonly more than 1000-fold. Similarly, antibody therapy in patients with a malignant disease employed as a monotherapy or in combination with chemotherapy reduces the number of malignant cells generally 10 to 100 fold, or more than 1000-fold. Therapy may be repeated over an extended amount of time to assure the complete elimination of infectious particles, malignant cells, etc. In some instances, therapy with antibody preparations will be continued for extended periods of time in the absence of detectable amounts of infectious particles or undesirable cells. Similarly, the use of antibody therapy for the modulation of immune responses may consist of single or multiple administrations of therapeutic antibodies. Therapy may be continued for extended periods of time in the absence of any disease symptoms.

The subject treatment may be employed in conjunction with chemotherapy at dosages sufficient to inhibit infectious disease or malignancies. In autoimmune disease patients or transplant recipients, antibody therapy may be employed in conjunction with immunosuppressive therapy at dosages sufficient to inhibit immune reactions.

The invention is further illustrated, but by no means limited, by the following examples.

20

### **Example 1**

#### **Novel Sequences 3'prime of the Cy Gene from Cows, Sheep and Rabbits**

25

Genomic DNA was isolated from blood of a Simmental cow using the QIAamp DNA Blood Kit (QIAGEN). The genomic region 3' of the cow Cy gene (i.e., the cow Cy gene 3' flanking sequence) was PCR-amplified using the isolated genomic DNA as template and the following primers:

30

5' primer: 5'cgcaagcttCCTACACGTGTGTGGTGATG3' (SEQ ID NO: 1);

3' primer: 5'cgcaagcttAAGATGGWGATGGTSGTCCA3' (SEQ ID NO: 2)(Universal degenerate code: W=(A/T) S=(G/C)).

The upper-case portion of the 5' primer was from exon 3 of Cy, and the lower-case portion represented a terminal HindIII restriction site. The upper-case portion of the 3' primer was a degenerate sequence designed according to the published sequences from the human M1 exon and the mouse M1 exon, and the lower-case portion represented a terminal HindIII restriction site. A 1.3kb PCR fragment was obtained using the EXPAND long template PCR system (Roche). The fragment was gel purified, digested with HindIII, and cloned into a Bluescript cloning vector. The resulting clones fell into three populations, which differ from one another in the pattern of the restriction fragments obtained with BamHI, EcoRI and XhoI. One clone from each population was sequenced, and the sequences are shown in Figure 1 (SEQ ID NOS: 3-5).

Genomic DNA was isolated from blood of a Merino sheep using the QIAamp DNA Blood Kit (QIAGEN). The genomic region 3' of the sheep Cy gene (i.e., the sheep Cy gene 3' flanking sequence) was PCR-amplified using the isolated genomic DNA as template and the following primers:

5' primer: 5'cgcgatccCCTACGCGTGTGTGGTGATG3' (SEQ ID NO: 6)

3' primer: 5'cgcgatccACCGAGGAGAAGATCCACTT3' (SEQ ID NO: 7)

The upper-case portion of the 5' primer was from exon 3 of Cy, and the lower-case portion represented a terminal BamHI restriction site. The upper-case portion of the 3' primer was designed according to the published sequences from the human M2 exon and the mouse M2 exon, and the lower-case portion represented a terminal BamHI restriction site. A 2.9kb PCR fragment was obtained using the EXPAND long template PCR system (Roche). The fragment was gel purified, digested with BamHI, and cloned into a Bluescript cloning vector. The resulting clones fell into two populations, which differ from each other in the pattern of the restriction fragments obtained with HindIII, EcoRI and XhoI. One clone from each population was sequenced, and the sequences are shown in Figure 2 (SEQ ID NOS: 8-9).

A 10kb EcoRI fragment containing the Cy gene and its flanking sequences from A2 allotype rabbit was subcloned from a genomic cosmid clone (cos 8.3 from

Knight et al., *J Immunol* (1985) 1245-50, "Organization and polymorphism of rabbit immunoglobulin heavy chain genes"). The nucleotide sequences 5' and 3' of C $\gamma$  were determined using standard methods and are set forth in Figure 3 and 5, SEQ ID NO: 10, 12, 13, respectively.

5                Sequences 3' of rabbit C $\kappa$ app1 were determined from an EcoRI/BamHI subclone from VJk2Ck In pSV2neo. The nucleotide sequence is set forth in Figure 4, SEQ ID NO: 11.

                 The amino acid sequences encoded by the M1 and M2 exons from cow, sheep and rabbit were deduced from the above 3' flanking sequence. These amino acid  
10 sequences were aligned with the published M1 and M2 sequences from camel, human and mouse, as shown in Figure 6.

### Example 2

#### **A Vector for Replacing the Rabbit Endogenous C $\gamma$ Gene Segment with the Human C $\gamma$ 1 Segment**

Genomic DNA is isolated from rabbit fetal fibroblasts of an a2-homozygous rabbit. The DNA sequence upstream of rabbit C $\gamma$  (i.e., the 5' flanking sequence of rabbit C $\gamma$ ) is amplified by PCR using the following primers:

20                5' taattatgcgccgcCTTCAGCGTGAACACGCCCTC 3' (SEQ ID NO: 39)  
with a 5' NotI site and

5' GTCGACGCCCCTCGATGCACTCCCAGAG 3' (SEQ ID NO: 40).

The DNA sequence downstream of rabbit C $\gamma$  (i.e., the 3' flanking sequence of rabbit C $\gamma$ ) is amplified with the following primers:

25                5' ggtaccCTCTCCCTCCCCACGCCGAGC 3' (SEQ ID NO: 41) with a 5' KpnI site and

5' atatctcagaACTGGCTGTCCCTGCTGTAGTACACGG 3' (SEQ ID NO: 42)  
with a 5' XhoI site.

Human genomic DNA is isolated from human peripheral blood lymphocytes.  
30 The DNA fragment encoding human C $\gamma$ 1 is amplified using the following primers:

5' GTCGACACTGGACGCTGAACCTCGCGG 3' (SEQ ID NO: 43) and

5' GGTACCGGGGGCTTGCCGGCCGTCGCAC 3' (SEQ ID NO: 44).

The fragments are digested with restriction enzymes and cloned into a Bluescript vector. Subsequently, a lox neo-cassette is inserted into the SalI site and an Hsv-tk cassette into the XhoI site. A schematic drawing of the final construct is shown in

5 Figure 7a.

### Example 3

#### **A Vector for Replacing the Rabbit Endogenous Cκ Gene Segment with the Human Cκ Segment**

10

Genomic DNA was isolated from rabbit fetal fibroblasts of a b5-homozygous rabbit. The DNA sequence upstream of rabbit Cκ1 (i.e., the 5' flanking sequence of rabbit Cκ1) was amplified by PCR using the following primers:

5' gcggccgcTGGCGAGGAGACCAAGCTGGAGATCAAACG 3' (SEQ ID  
15 NO: 45) with a 5' NotI site

5' GTCGACGCAGCCCAAAGCTGTTGCAATGGGGCAGCG 3' (SEQ ID  
NO: 46).

The DNA sequence downstream of rabbit Cκ1 (i.e., the 5' flanking sequence of rabbit Cκ1) was amplified with the following primers:

20 5' atatgtaccGCGAGACGCCTGCCAGGGCACCGCC 3' (SEQ ID NO: 47)  
with a 5' KpnI site

5' GGATCCCGAGCTTTATGGGCAGGGTGGGGG 3' (SEQ ID NO: 48).

Human genomic DNA was isolated from human peripheral blood lymphocytes. The DNA fragment encoding human Cκ was amplified using the following  
25 primers:

5' ATATGTCGACCTGGGATAAGCATGCTGTTTTCTGTCTGTCCC 3'  
(SEQ ID NO: 49)

5' CTAGGTACCAGCAGGTGGGGGCACTTCTCCC 3' (SEQ ID NO: 50).

The fragments were digested with restriction enzymes and cloned into a Bluescript vector.  
30 Subsequently, a lox neo-cassette was inserted into the SalI site and an Hsv-tk cassette into the XhoI site. A schematic drawing of the final construct is shown in Figure 7b.

#### **Example 4**

##### **Replacement of the Endogenous $C\gamma$ and $C\kappa$ Gene Segments in Rabbit Fetal Fibroblasts with the Corresponding Human Gene Segments**

5 Rabbit fetal fibroblast cells are prepared by standard methods. After one passage, fibroblasts are transfected with 5 $\mu$ g of the NotI-linearized targeting vector as shown in Figure 5a for  $C\gamma$  or Figure 51b for  $C\kappa$ , and are seeded in 96-well plates (2 x 10<sup>3</sup> cells/well). After a positive selection with 600 $\mu$ g/ml G418 and a negative selection with  
10 200nM FIAU, resistant colonies are replica-plated to two 96-well plates for DNA analysis and cryopreservation, respectively. PCR and/or Southern blot analysis is performed to identify cells with the human  $C\gamma 1$  gene segment integrated in the genome. The cells having the integrated human  $C\gamma 1$  gene are used in rabbit cloning as described in Example 5.

15

#### **Example 5**

##### **Cloning of Rabbits**

Mature Dutch Belton rabbits are superovulated by subcutaneous injection of  
20 follicle stimulating hormone (FSH) every 12 hours (0.3 mg x 2 and 0.4 mg x 4). Ovulation is induced by intravenous administration of 0.5 mg luteinizing hormone (LH) 12 hours after the last FSH injection. Oocytes are recovered by ovidual flush 17 hours after LH injection. Oocytes are mechanically enucleated 16-19 hours after maturation. Chromosome removal is assessed with bisBENZIMIDE (HOECHST 33342, Sigma, St.  
25 Louis, MO) dye under ultraviolet light. Enucleated oocytes are fused with actively dividing fibroblasts by using one electrical pulse of 180 V/cm for 15  $\mu$ s (Electrocell Manipulator 200, Genetronics, San Diego, CA). After 3-5 hours oocytes are chemically activated with calcium ionophore (6  $\mu$ M) for 4 min (# 407952, Calbiochem, San Diego, CA) and 2 mM 6-dimethylaminopurine (DMAP, Sigma) in CR2 medium (Specialty  
30 Media, Lavalett, NJ) with 3 mg/ml bovine serum albumin (fatty acid free, Sigma) for 3 hours. Following the activation, the embryos are washed in hamster embryo culture

medium (HECM)-Hepes five times and subsequently, cultivated in CR2 medium containing 3 mg/ml fatty-acid free BSA for 2-48 hours at 37.8° C and 5%CO<sub>2</sub> in air. Embryos are then transferred into synchronized recipients. Offsprings are analyzed by PCR for a segment of the transgene.

5

### Example 6

#### **Construction of a DNA Fragment Containing a Portion of a Rabbit Heavy Chain Locus with a Human Cy1 Gene Segment and a VH Gene Segment Encoding a Human VH Domain Polypeptide Sequence**

10

The upstream and downstream regions (i.e., the 5' and 3' flanking regions) of the rabbit heavy chain Cy gene from an a2-allotype rabbit were sequenced. A DNA fragment (SEQ ID NO: 51) is generated by PCR using overlapping oligonucleotides wherein the DNA fragment contains from 5' to 3', a sequence derived from the 5' flanking region of the rabbit Cy gene, the human Cy1 gene, and a sequence derived from the 3' flanking region of the rabbit Cy gene (Figure 8).

15

A genomic BAC library derived from an a2-allotype rabbit is generated by standard procedures and screened with probes specific for rabbit Cy. A BAC clone containing rabbit heavy chain gene segments is identified. The rabbit Cy gene on this BAC clone is replaced with the human Cy1 gene by homologous recombination in *E.coli* using the DNA fragment of SEQ ID NO: 51 and the pET system. This replacement is accomplished by two consecutive recombination steps: first the rabbit Cy gene segment is replaced with a marker gene; then the marker gene is replaced the human Cy1 gene segment.

20

25

The modified BAC clone containing rabbit heavy chain genes and the inserted human Cy1 gene is further modified by replacing the 3'proximal VH1 segment with a synthetic VH gene segment (Figure 9). This synthetic VH gene segment (SEQ ID NO: 52) is made using overlapping oligonucleotides and includes a 5' flanking sequence, a 3' flanking sequence, and a sequence coding for a polypeptide nearly identical to the human immunoglobulin heavy chain variable domain polypeptide sequence described by Huang

30

and Stollar (*J. Immunol.* 151: 5290-5300, 1993). The coding sequence of the synthetic VH gene segment is designed based on the published sequence of a rabbit VH1 gene ( $\alpha 2$ , Knight and Becker, *Cell* 60:963-970, 1990) and is more than 80% identical to rabbit VH gene segments. The 5' and the 3' flanking sequences in the synthetic VH segment are  
5 derived from the upstream and downstream regions of the  $\alpha 2$ -allotype rabbit VH1 gene. The synthetic VH gene of SEQ ID NO: 52 is used to replace the rabbit VH1 gene on the BAC clone by homologous recombination using the pET or the  $\text{red}\beta\gamma$  system. The modified BAC clone is amplified and purified using standard procedures.

10

#### Example 7

#### **Construction of a DNA Fragment Containing a Portion of a Rabbit Light Chain Locus with a Human C $\kappa$ Gene Segment and a VJ Gene Segment Encoding a Human VL Domain Polypeptide Sequence**

15

The upstream and downstream regions (i.e., the 5' and 3' flanking regions) of the rabbit light chain C $\kappa$ 1 gene from a b5-allotype rabbit were sequenced. A DNA fragment (SEQ ID NO: 53) is generated by PCR using overlapping oligonucleotides wherein the DNA fragment contains from 5' to 3', a sequence derived from the 5' flanking region of the rabbit C $\kappa$ 1 gene, the human C $\kappa$ 1 gene, and a sequence derived from  
20 the 3' flanking region of the rabbit C $\kappa$ 1 gene (Figure 10).

A genomic BAC library derived from a b5-allotype rabbit is generated by standard procedures and screened with probes specific for rabbit C $\kappa$ 1. A BAC clone containing rabbit light chain gene segments is identified. The rabbit C $\kappa$ 1 gene on this  
25 BAC clone is replaced with the human C $\kappa$ 1 gene on the DNA fragment of SEQ ID NO: 53 by homologous recombination in *E.coli* using the pET or the  $\text{red}\beta\gamma$  system. This replacement is accomplished by two consecutive recombination steps: first the rabbit C $\kappa$ 1 gene segment is replaced with a marker gene; then the marker gene is replaced the human C $\kappa$ 1 gene segment.

30 The modified BAC clone containing rabbit light chain genes and the inserted human C $\kappa$ 1 gene is further modified by inserting a rearranged VJ DNA fragment into the

J region of the rabbit light chain locus. The rearranged VJ DNA fragment encodes a human immunoglobulin variable domain polypeptide described by Pritsch et al. (*Blood* 82(10):3103-3112, 1993) and Lautner-Rieske et al. (*Eur. J. Immunol.* 22 (4), 1023-1029, 1992)) (Figure 7). The nucleotide sequence of the rearranged VJ fragment is designed to maximize the sequence homology at the nucleotide level to the rabbit Vkappa sequence published by Lieberman et al. (*J. Immunol.* 133 (5), 2753-2756, 1984). This rearranged VJ DNA sequence is more than 80% identical with known rabbit V $\kappa$  genes. Using overlapping oligonucleotides in PCR, the rearranged VJ DNA fragment is linked to a 5' and a 3' flanking sequence, resulting the DNA fragment of SEQ ID NO: 54 (Figure 11). The 5' flanking sequence is derived from 5' of a rabbit V $\kappa$ , the 3' flanking sequence is derived from 3' of rabbit J2. The DNA fragment of SEQ ID NO: 54 is subsequently inserted into the rabbit light chain locus by homologous recombination in *E. coli* using the pET or the red $\beta$ y system. The insertion is performed in such a way that the rabbit light chain region containing the rabbit V $\kappa$ 1 gene segment, the rabbit J1 and J2 segments, and the sequences in between, is replaced with the rearranged VJ DNA fragment. Again, this insertion is accomplished by replacement of the rabbit V to J region with a marker gene, followed by the replacement of the marker gene with the rearranged VJ DNA fragment. The modified BAC clone is amplified and purified using standard procedures.

20

### Example 8

#### **Transgenic Rabbits Expressing the Humanized Immunoglobulin Light and/or Heavy Chain Transgene**

Transgenic rabbits are generated as described by Fan et al. (*Pathol. Int.* 49: 583-594, 1999). Briefly, female rabbits are superovulated using standard methods and mated with male rabbits. Pronuclear-stage zygotes are collected from oviduct and placed in an appropriate medium such as Dulbecco's phosphate buffered saline supplemented with 20% fetal bovine serum. The exogenous DNA (e.g., the humanized BAC clone from Example 4 and/or 5 which has been linearized prior to injection) is microinjected into the male pronucleus with the aid of a pair of manipulators. Morphological surviving zygotes

30

are transferred to the oviducts of pseudopregnant rabbits. Pseudopregnancy is induced by the injection of human chorionic gonadotrophin (hCG). Between about 0.1-1% of the injected zygotes develop into live transgenic rabbits. Integration of the transgene in the genome is confirmed by Southern blots analysis using a probe specific for the transgene.

5 cDNA is prepared using RNA isolated from B cells (in blood, spleen and/or lymph nodes) of a transgenic rabbit. Primers specific for the human transgene (human CH gene segment or the synthetic humanized VH gene segment) are used to generate amplified products from cDNA. The observation of amplified products indicates that the transgene is rearranged in the transgenic animal and the rearranged transgene is  
10 transcribed in the animal. Amplified products are sequenced and the presence of donor sequences from upstream V genes indicates that the transgene introduced into the germline of the animal undergoes gene conversion.

The presence of antibodies containing human IgG and/or human kappa light chain antigenic determinants in the serum of transgenic founder rabbits is determined  
15 using an ELISA assay.

20

### Example 9

#### **Production of Humanized Antibodies From Transgenic Rabbits with the Genetic Background of the Alicia and/or Basilea Rabbit Strain**

25 The Alicia strain lacks the VH1 gene segment and therefore has an impaired Ig heavy chain expression. Transgenic founder rabbits capable of expressing humanized heavy chain molecules in the genetic background of the Alicia rabbit strain are generated, e.g., by using fetal fibroblasts established from Alicia rabbits in Examples 4-5 above, or by using zygotes from female Alicia rabbits mated with male Alicia rabbits in Example 8  
30 above. Transgenic animals are also obtained which are homozygous for the Alicia Ig phenotype and are also homozygous for a humanized heavy chain transgene. Serum is tested in ELISA for the presence of humanized heavy chain (e.g., a human heavy chain

constant region). The concentration of antibodies with humanized Ig heavy chains in these homozygous Alicia animals is substantially higher, e.g., about 10 to 100 fold higher, than that produced from a transgene integrated in the genome of wild type (non-Alicia) rabbits.

5           The Basilea strain does not express  $\kappa 1$  light chain and in its place exclusively express the  $\kappa 2$  and  $\lambda$  light chains. Transgenic founder rabbits capable of expressing humanized light chain molecules in the genetic background of the Basilea rabbit strain are generated, e.g., by using fetal fibroblasts established from Basilea rabbits in Examples 4-5 above, or by using zygotes from female Basilea rabbits mated with male Basilea rabbits in  
10   Example 8 above. Transgenic animals are obtained which are homozygous for the Basilea light chain phenotype, and are also homozygous for a humanized light chain transgene. Serum is tested in ELISA for the presence of the humanized light chain. The concentration of the humanized light chain in the homozygous Basilea animals is substantially higher, about 10-100 fold higher, than the concentration of a humanized light  
15   chain in a transgenic rabbit with the wild type (non-Basilea) genetic background. Transgenic founder rabbits are mated with each other to generate transgenic rabbits with the following traits: (1) having at least one humanized light chain transgene, (2) having at least one humanized heavy chain transgene, (3) homozygous for the Alicia heavy chain locus, and (4) homozygous for the Basilea light chain locus.

20

#### **Example 10**

#### **Construction of a DNA Fragment Containing a Modified Chicken Light Chain Locus Having a Human Clambda2 Gene Segment and a VJ Gene Segment Encoding a Human VL Domain**

25

A genomic BAC library derived from a jungle fowl chicken was screened with radiolabeled probes specific for chicken light chain Clambda and chicken Vpsi25 (the V gene segment at the very 5' end of the light chain locus). A BAC clone containing the entire lambda light chain locus was identified. The chicken C $\lambda$  gene on this BAC clone is  
30   replaced with the human C $\lambda 2$  gene by homologous recombination in *E. coli* using the pET system (Zhang et al., *Nat. Biotechnol.* 18(12):1314-7, 2000) as follows.

A first DNA fragment containing a kanamycin selection cassette was generated by PCR using primers specific for Tn5 gene. The 5' primer (5'catacacagccatacatagcggtgtggccgctctgcctctctcttcaggTATGGACAGCAAGCGAACCG 3', SEQ ID NO: 55) was designed to include 50 bp at the 5' end (lower case), derived from the 5' flanking region of the chicken light chain C $\lambda$  gene. The 3' primer (5'atcagggtgacccctacgttacactcctgtcaccaaggagtgggaggacTCAGAAGAACTCGTCAAGA AG3', SEQ ID NO: 56) was designed to include about 50 bp at the end (lower case), derived from the 3' flanking region of the chicken light chain C $\lambda$  gene.

A second DNA fragment (SEQ ID NO: 57) was synthesized using overlapping oligonucleotides wherein the DNA fragment contains from 5' to 3', a sequence derived from the 5' flanking region of the chicken light chain Clambda gene, the human Clambda2 gene, and a sequence derived from the 3' flanking region of the chicken Clambda gene (Figure 12).

*E. coli* cells of the chicken light chain BAC clone were transformed with a recombination plasmid expressing the recE and recT functions under an inducible promotor. Cells transformed with the recombination plasmid were then transformed with the first DNA fragment above and selected afterwards in media containing kanamycin. Clones resistant to kanamycin were identified, and the replacement of the chicken C $\lambda$  segment by the kanamycin selection cassette via homologous recombination was confirmed by restriction enzyme digest.

In the second homologous recombination step, cells positive for the kanamycin selection cassette were transformed with the second DNA fragment above. Transformed cells were screened for the loss of kanamycin resistance as indicative of the replacement of the kanamycin selection cassette by the human C $\lambda$ 2 gene. The exchange was confirmed by restriction enzyme digest and/or sequence analysis.

The ET cloning procedure is summarized in Figure 13.

The BAC clone containing the chicken light chain locus and the inserted human Clambda2 gene segment was further modified by inserting a rearranged VJ DNA fragment. The rearranged VJ DNA fragment encodes a human immunoglobulin variable domain polypeptide described by Kametani et al. (*J. Biochem.* 93 (2), 421-429, 1983) as

IG LAMBDA CHAIN V-I REGION NIG-64 (P01702) (Figure 14). The nucleotide sequence of the rearranged VJ fragment was so designed as to maximize the sequence homology at the nucleotide level to the chicken Vlamba1 sequence published by McCormack et al. (*Cell* 56, 785-791, 1989). This rearranged VJ DNA sequence is more than 80% identical with known chicken light chain V genes. The rearranged VJ DNA fragment was linked to a 5' flanking sequence and a 3' flanking sequence, resulting in the DNA fragment of SEQ ID NO: 58 (Figure 14). The 5' flanking sequence was derived from 5' of chicken Vlamba1, and the 3' flanking sequence was derived from 3' of chicken J. The DNA fragment of SEQ ID NO: 58 was subsequently inserted into the chicken light chain locus in *E. coli* using the pET system as shown in Figure 15. The insertion was performed in such a way that the region on the chicken light chain locus from the 5' end of the chicken Vlamba1 gene segment to the 3' end of the chicken J region was replaced with the rearranged, synthetic VJ DNA fragment. Again, this insertion was accomplished by the replacement of the chicken V-J region with a marker gene, followed by the replacement of the marker gene with the rearranged VJ DNA fragment. The modified region of the chicken light chain locus is shown in Figure 15. The modified BAC clone was amplified and purified using standard procedures.

#### Example 11

##### **Construction of a DNA Fragment Containing a Portion of a Chicken Heavy Chain Locus With a Human Cy1 Gene Segment and a VH Gene Segment Encoding a Human VH Domain Polypeptide Sequence**

A jungle fowl chicken genomic BAC library was generated by standard procedures and screened with probes specific for chicken Cy. A BAC clone containing chicken heavy chain gene segments is identified. The upstream and downstream regions (i.e., the 5' and 3' flanking regions) of the heavy chain Cy gene are sequenced. The chicken Cy gene on this BAC clone is replaced with the human Cy1 gene by homologous recombination in *E. coli* using the pET system as follows.

A first DNA fragment containing a kanamycin selection cassette is generated by PCR using primers specific for Tn5 gene. The 5' and 3' primers are designed to

include about 50 bp at the end, derived from the 5' and 3' flanking regions of the chicken heavy chain Cy gene.

A second DNA fragment is generated by PCR using overlapping oligonucleotides wherein this second DNA fragment contains from 5' to 3', a sequence of about 50 bp derived from the 5' flanking region of the chicken Cy gene, the human Cy1  
5 gene, and a sequence of about 50 bp derived from the 3' flanking region of the chicken Cy gene.

*E. coli* cells of the chicken CY BAC clone are transformed with a recombination plasmid expressing the recE and recT functions under an inducible  
10 promotor. Cells transformed with the recombination plasmid are further transformed with the first DNA fragment and selected in media containing kanamycin. Clones resistant to kanamycin are identified, and the replacement of the chicken CY segment by the kanamycin selection cassette via homologous recombination is confirmed by restriction enzyme digest.

15 In the second homologous recombination step, cells positive for the kanamycin selection cassette are now transformed with the second DNA fragment described above. Transformed cells are screened for loss of kanamycin resistance as indicative of the replacement of the kanamycin selection cassette by the human Cy1 gene. The exchange is confirmed by restriction enzyme digest and/or sequence analysis.

20 The BAC clone containing the inserted human Cy1 gene is further modified by replacing the 3'proximal VH1 segment (i.e., the 3'proximal VH1 gene in the V region) with a synthetic VH gene segment. This synthetic VH gene segment is designed based on the published sequence of a chicken VH1 gene (Arakawa et al., EMBO J 15(10): 2540-2546, 1996). The synthetic gene segment is more than 80% identical to chicken VH gene  
25 segments and encodes an amino acid sequence that is identical to the amino acid sequence of a human immunoglobulin heavy chain variable domain polypeptide described by Matthyssens and Rabbitss (in Steinberg CM and Lefkovits I, (eds). *The Immune System*: 132-138, S. Karger, NY 1981). This synthetic VH segment including 5' and 3' flanking sequences is synthesized by PCR using overlapping oligonucleotides. The 5' and the 3'  
30 flanking sequences are derived from the upstream and downstream regions of chicken

VH1 gene. This synthetic VH segment is used to replace the chicken VH1 gene on the BAC clone by homologous recombination using the pET system. The modified BAC clone is amplified and purified using standard procedures.

5

### Example 12

#### **Transgenic Chicken Expressing the Humanized Immunoglobulin Light and/or Heavy Chain Transgenes**

The production of transgenic chicken is carried out using techniques as described by Etches et al., *Methods in Molecular Biology* 62: 433-450; Pain et al., *Cells Tissues Organs* 1999; 165(3-4): 212-9; Sang, H., "Transgenic chickens--methods and potential applications", *Trends Biotechnol* 12:415 (1994); and in WO 200075300, "Introducing a nucleic acid into an avian genome, useful for transfecting avian blastodermal cells for producing transgenic avian animals with the desired genes, by directly introducing the nucleic acid into the germinal disc of the egg".

Briefly, the modified BAC clones are linearized and mixed with a transfection reagent to promote uptake of DNA into cells. The formulations are injected into a multicell stage chicken embryo in close proximity to the germinal disc. The window in the egg shell is closed and the eggs are incubated. After hatching chimeric chickens are identified by PCR and Southern blot analysis using transgene specific sequences. Integration of the transgene in the genome is confirmed by Southern blots analysis using a probe specific for the transgene. Heavy and light chain transgenic animals are bred with each other to generate transgenic chickens expressing antibodies having humanized heavy and light chains.

cDNA is prepared using RNA isolated from B cells (in blood, spleen and/or lymph nodes) from transgenic chickens. Primers specific for the human transgene (e.g., human CH gene segments and/or the synthetic humanized VH gene segments) are used to generate amplified products from cDNA. The observation of amplified products indicates that the transgene is rearranged in the transgenic animal and the rearranged transgene is transcribed in the animal. Amplified products are sequenced and the presence of donor

30

sequences from upstream V genes indicates that the transgene introduced into the germline of the animal undergoes gene conversion.

The presence of antibodies containing human IgG and/or human kappa light chain antigenic determinants in the serum of transgenic chickens is determined using an ELISA assay.

### **Example 13**

#### **Production of Functional Humanized Antibodies in Transgenic Chicken with the Agammaglobulinemic Phenotype**

10

Transgenic chickens with the following traits are produced: (1) having at least one humanized light chain transgene, (2) having at least one humanized heavy chain transgene, and (3) homozygous for the agammaglobulinemic phenotype. These animals produce antibodies into the blood and eggs, and antibodies can be purified from either source. In general, antibody concentrations in the eggs are about 5% to 50% of antibodies concentration in the blood. Animals that contain humanized antibodies at high levels in eggs can be selected and bred to produce offspring. Alternatively, transgenic animals can be generated that specifically secrete humanized antibodies into their eggs.

20

### **Example 14**

#### **Generation Of Transgenic Chickens Expressing Humanized Immunoglobulin**

25

Chicken embryonic stem cells are isolated and cultured as described by Pain et al. (*Development* 122, 2339-2348; 1996). Chicken embryos are obtained from eggs immediately after they are laid. The entire blastoderm is removed by gentle aspiration, embryos are slowly dissociated mechanically and cells are seeded in ESA complete medium on inactivated STO feeder cells. ESA medium is composed of MEM medium containing 10% FCS, 2% chicken serum, 1% bovine serum albumin, 10 ng/ml ovalbumin, 30 1 mM sodium pyruvate, 1% non-essential amino acids, 1  $\mu$ M of each nucleotide

adenosine, guanosine, cytidine, uridine, thymidine, 0.16 mM  $\beta$ -mercaptoethanol, ESA complete medium is supplemented with 10 ng/ml bFGF, 20 ng/ml h-IGF-1, 1% vol/vol avian-SCF and 1% vol/vol h-LIF, 1% vol/vol h-IL-11. Cell cultures are incubated at 37°C in 7.5 CO<sub>2</sub> and 90% humidity. After 48 hours fresh blastodermal cells are added to the culture in half of the original volume of ESA complete medium. After an additional incubation for three days, the culture medium is partially (50%) replaced with fresh ESA complete medium, and totally every day thereafter. For cell harvesting, cultures are washed with PBS and incubated in a pronase solution (0.025% w/v). Dissociated cells are transfected with various linearized transgenic constructs containing a humanized Ig locus. Transfected cells are incubated with STO feeder cells (as described above) in the presence of selective antibiotics. Cells are transferred onto fresh feeder cells twice per week. Antibiotic resistant cells are isolated and the integration of a humanized Ig gene fragments at a random site or at the corresponding chicken immunoglobulin gene loci is confirmed by PCR.

Subsequently, genetically modified cells are injected into recipient embryos. As recipient embryos, freshly laid eggs are irradiated (6Gy - Cobalt source). Between 100 to 200 genetically modified cells are injected into the subgerminal cavity using a micropipet. The window in the egg shell is closed and the eggs are incubated. Somatic chimerism of hatched chickens is evaluated by PCR. Germ-line chimerism is assessed by mating of somatic chimeras.

### **Example 15**

#### **Immunization Of Transgenic Animals**

Genetically engineered chickens are immunized intramuscularly with purified Hepatitis B surface antigen (HBsAg) (5 $\mu$ g in incomplete Freund's adjuvant) on day 0, 14 and day 28. On day 35 animals are bled and serum is prepared. ELISA plates (NUNC, Denmark) are coated with 1  $\mu$ g/ml HBsAg in PBS for 1 hour at room temperature. Subsequently, available binding sites are blocked by incubation with 1% non-fat dry milk (NFM) in PBS (300  $\mu$ l/well). Chicken serum is diluted in PBS/1%NFM and added to the

coated wells. After an incubation of 1 hour, the plates are washed 3 times with PBS/0.05% Tween 20 and bound Ig is detected using goat anti-human Ig conjugated with horseradish peroxidase. Conjugated goat antibody is detected using o-phenylenediamine dihydrochloride (Sigma) at 1 mg/ml. The colorimetric reaction is stopped by addition of 1 M HCl solution and the absorbance is measured at 490 nm. As a control, serum from non-immunized chicken is used. Serum from non-immunized chickens does not react with HBsAg. At a dilution of 1:250 the optical density measured in uncoated and HBsAg coated wells is below 0.2. In contrast, serum from immunized chickens contains humanized antibodies reactive with HBsAg. At a serum dilution of 1:250 the measured optical density is 2.3. Upon further dilution of the serum the measured optical density declines to 0.1 (at a dilution of 25600). No antibodies reactive with a goat anti-chicken IgG-HRP conjugate can be detected. This demonstrates that the genetically engineered chickens produce humanized anti-HBsAg antibodies following immunization.

Genetically engineered rabbits are immunized intramuscularly with purified Hepatitis B surface antigen (HBsAg) (10µg in incomplete Freund's adjuvant) on day 0 and day 14. On day 28 animals are bled from the ear and serum is prepared. ELISA plates (NUNC, Denmark) are coated with 1 µg/ml HBsAg in PBS for 1 hour at room temperature. Subsequently, available binding sites are blocked by incubation with 1% non-fat dry milk (NFM) in PBS (300 µl/well). Rabbit serum is diluted in PBS/1%NFM and added to the coated wells. After an incubation of 1 hour, the plates are washed 3 times with PBS/0.05% Tween 20 and bound Ig is detected using goat anti-human Ig conjugated with horse-radish peroxidase. Conjugated goat antibody is detected using o-phenylenediamine dihydrochloride (Sigma) at 1 mg/ml. The colorimetric reaction is stopped by addition of 1 M HCl solution and the absorbance is measured at 490 nm. As a control serum from non-immunized rabbits is used. Serum from non-immunized rabbits does not react with HBsAg. At a dilution of 1:100 the optical density measured in uncoated and HBsAg coated wells is below 0.4. In contrast, serum from immunized rabbits contains partially human antibodies reactive with HBsAg. At a serum dilution of 1:100 the measured optical density is 2.8. Upon further dilution of the serum the measured optical density declines to 0.2 (at a dilution of 25600). No antibodies reactive

with a goat anti-rabbit IgG-HRP conjugate can be detected. This demonstrates that the genetically engineered rabbits produce humanized anti-HBsAg antibodies following immunization.

5

#### **Example 16**

##### **Complement Mediated Cytotoxicity of Virus Infected Cell Line Using Humanized Antibodies**

A human liver carcinoma cell line expressing HBsAg is labeled with 0.1 mCi  
10  $^{51}\text{Cr}$  in 100  $\mu\text{l}$  PBS for 1 hr at 37°C. Two thousand  $^{51}\text{Cr}$ -labeled cells are incubated with  
serum from genetically engineered rabbits or chickens expressing anti-HbsAg humanized  
immunoglobulins. After two hours at 37°C the release of  $^{51}\text{Cr}$  into the supernatant is  
determined by measuring radioactivity using a scintillation counter. For the determination  
of maximum release, 1% Triton X100 is added. The degree of cell lysis is calculated as  
15 follows:  $\% \text{Lysis} = \text{CPM}_{\text{experimental}} \pm \text{CPM}_{\text{spontaneous}} / \text{CPM}_{\text{total}} \pm \text{CPM}_{\text{spontaneous}}$ . Incubation of labeled cells with serum (diluted 1:30) from non-immunized  
rabbits does not result in cell lysis (<10%). However, incubation of cells with serum from  
immunized rabbits causes 80% cell lysis. Inactivation of complement in the serum by  
heat treatment (56°C for 30 minutes) renders the serum from immunized rabbits inactive.  
20 These results demonstrate that humanized antibodies produced by genetically engineered  
rabbits bind to HBsAg-positive cells and cause complement dependent lysis.

#### **Example 17**

##### **Immunization of Transgenic Animals against Staphylococcus aureus**

25

Genetically engineered chickens are immunized intramuscularly with a  
recombinant fragment of the Staphylococcus aureus collagen adhesin protein (100 $\mu\text{g}$  in  
incomplete Freund's adjuvant) on day 0, 14 and day 28. On day 35 animals are bled and  
serum is prepared. ELISA plates (NUNC, Denmark) are coated with 2  $\mu\text{g}/\text{ml}$  collagen  
30 adhesin protein in PBS for 1 hour at room temperature. Subsequently, available binding  
sites are blocked by incubation with 1% non-fat dry milk (NFM) in PBS (300  $\mu\text{l}/\text{well}$ ).

Chicken serum is diluted in PBS/1%NFM and added to the coated wells. After an incubation of 1 hour, the plates are washed 3 times with PBS/0.05% Tween 20 and bound Ig is detected using goat anti-human Ig conjugated with horseradish peroxidase. Conjugated goat antibody is detected using o-phenylenediamine dihydrochloride (Sigma) at 1 mg/ml. The colorimetric reaction is stopped by addition of 1 M HCl solution and the absorbance is measured at 490 nm. As a control, serum from non-immunized chicken is used. Serum from non-immunized chickens does not react with collagen adhesin protein. At a dilution of 1:250 the optical density measured in uncoated and collagen adhesin protein coated wells is below 0.2. In contrast, serum from immunized chickens contains humanized antibodies reactive with collagen adhesin. At a serum dilution of 1:250 the measured optical density is 2.3. Upon further dilution of the serum the measured optical density declines to 0.1 (at a dilution of 25600). No antibodies reactive with a goat anti-chicken IgG-HRP conjugate can be detected. This demonstrates that the genetically engineered chickens produce humanized anti-Staph. aureus collagen adhesin antibodies following immunization.

Genetically engineered rabbits are immunized intramuscularly with recombinant fragment of the Staphylococcus aureus collagen adhesin protein (100µg in incomplete Freund's adjuvant) on day 0 and day 14. On day 35 animals are bled and serum is prepared. ELISA plates (NUNC, Denmark) are coated with 2 µg/ml collagen adhesin protein in PBS for 1 hour at room temperature. Subsequently, available binding sites are blocked by incubation with 1% non-fat dry milk (NFM) in PBS (300 µl/well). Rabbit serum is diluted in PBS/1%NFM and added to the coated wells. After an incubation of 1 hour, the plates are washed 3 times with PBS/0.05% Tween 20 and bound Ig is detected using goat anti-human Ig conjugated with horseradish peroxidase. Conjugated goat antibody is detected using o-phenylenediamine dihydrochloride (Sigma) at 1 mg/ml. The colorimetric reaction is stopped by addition of 1 M HCl solution and the absorbance is measured at 490 nm. As a control, serum from non-immunized rabbit is used. Serum from non-immunized rabbits does not react with collagen adhesin protein. At a dilution of 1:250 the optical density measured in uncoated and collagen adhesin protein coated wells is below 0.2. In contrast, serum from immunized rabbits contains

humanized antibodies reactive with collagen adhesin. At a serum dilution of 1:250 the measured optical density is 2.3. Upon further dilution of the serum the measured optical density declines to 0.1 (at a dilution of 25600). No antibodies reactive with a goat anti-rabbit IgG-HRP conjugate can be detected. This demonstrates that the genetically engineered rabbits produce humanized anti-Staph. aureus collagen adhesin antibodies following immunization.

### **Example 18**

#### **Protection Against *Staphylococcus Aureus* Infection In A Mouse Model**

10

Naive mice are passively immunized i.p. on day -1 with 16 mg of the immunoglobulin fraction containing antibodies specific for the *S. aureus* collagen adhesin protein (from Example 17) or with the immunoglobulin fraction from non-immunized animals. On day 0, the mice are challenged i.v. with  $4 \times 10^7$  CFU *S. aureus* per mouse and mortality is monitored over the next 7 days. Mortality rate in the control groups is 80% and 10% in the group treated with the immunoglobulin fraction containing antibodies specific for the *S. aureus* collagen adhesin protein. The data indicate that anticollagen adhesin antibodies can protect mice against lethal *S. aureus* challenge.

20

### **Example 19**

#### **Antigen-Specific Hybridomas Made From Transgenic Animals.**

Transgenic animals are immunized with an antigen (e.g., KLH, human red blood cells or sheep red blood cells). Spleen cells are removed at various times after immunization and fused with myeloma cell lines derived from rabbit and chicken, respectively. After fusion cells are plated into 96 well plates and supernatants are tested for the presence of humanized antibodies. To demonstrate that the antibodies contain human immunoglobulin sequences, hybridomas are stained with fluorescent-labeled antibodies reactive with human heavy and light chain immunoglobulins. Limiting dilution is conducted to purify hybridomas to monoclonality.

30

**Example 20****Evaluation of Immunogenicity**

5           Serum samples are collected from five cynomologous monkeys on day 0. Subsequently, a purified partially human polyclonal antibody preparation (5 mg/kg) is administered into five cynomologous monkeys by intravenous administration. The administration is repeated six times in bi-weekly intervals. Monkeys are monitored closely for any side-effects (e.g., anaphylactic shock, reflected by an elevated body  
10 temperature). After seven months serum is collected from blood samples. Affinity resins containing purified human IgG or partially human IgG are produced by standard procedure using CNBr-activated Sepharose. Monkey serum samples (3 ml) are added to the IgG-affinity resin (4 ml) containing 10 mg human or partially human IgG. Subsequently, the columns are washed with PBS. Bound monkey immunoglobulin is  
15 eluted from the column with 0.1M glycyl/HCl pH2.5 and dialyzed 2 times against PBS. The protein content of the eluted fractions is determined using the BCA assay using human IgG as a standard. The total amounts of protein in these fractions demonstrate that therapy with partially human IgG does not lead to a significant antibody response in the treated animals.

**Example 21****Treating Animals Using Humanized Antibodies**

Humanized polyclonal immunoglobulins are purified from the serum of genetically engineered rabbits, or from egg yolk of genetically engineered chickens, by  
25 ammonium sulfate precipitation and ion exchange chromatography. SCID-mice are injected with one million human liver carcinoma cells expressing HBsAg. Subsequently, 25 µg immunoglobulin is injected peritoneally once per day. Animals treated with antibodies isolated from non-immunized rabbit serum die after about 60 days. This is similar to untreated recipients of liver carcinoma cells. In contrast, mice treated with  
30 antibodies isolated from immunized rabbit serum survive for more than 150 days. This

demonstrates that human antibodies produced in genetically engineered rabbits are capable of eliminating human carcinoma cells from SCID-mice.

**What is claimed is:**

1. An isolated nucleic acid molecule comprising the sequence as set forth in any one of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 9,  
5 SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, or SEQ ID NO: 13, or a portion of any one of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, or SEQ ID NO: 13.
- 10 2. A recombination vector for replacing an Ig gene segment from a non-human animal with a human Ig gene segment, comprising from 5' to 3', a 5' nucleotide sequence, said human Ig gene segment, and a 3' nucleotide sequence, wherein said 5' nucleotide sequence and said 3' nucleotide sequence are homologous to the 5' and 3' flanking sequences of said Ig gene segment from the non-human animal.
- 15 3. The recombination vector of claim 2, wherein said non-human animal is an animal which relies primarily on gene conversion in generating antibody diversity.
4. The recombination vector of claim 3, wherein said animal is rabbit, pig, chicken,  
20 sheep or cow.
5. The recombination vector of claim 3, wherein the Ig gene segment from a non-human animal is a gene segment coding for a heavy chain or light chain constant region.
- 25 6. The recombination vector of claim 5, wherein said vector comprises from 5' to 3', a 5' nucleotide sequence as set forth in any one of SEQ ID NO: 12, SEQ ID NO: 13, a portion of SEQ ID NO: 12, or a portion of SEQ ID NO: 13; a human heavy chain constant region gene segment; a 3' nucleotide sequence as set forth in SEQ

ID NO: 10 or a portion of or SEQ ID NO: 10; and wherein said vector is useful for replacing a rabbit heavy chain constant region gene segment.

- 5 7. The recombination vector of claim 5, comprising the nucleotide sequence as set forth in SEQ ID NO: 51 wherein said vector is useful for replacing a rabbit heavy chain constant region gene segment.
8. The recombination vector of claim 5, wherein said vector is useful for replacing a rabbit light chain constant region gene and comprises a nucleotide sequence as set  
10 forth in SEQ ID NO: 53.
9. The recombination vector of claim 5, wherein said vector is useful for replacing a chicken light chain constant region gene and comprises a nucleotide sequence as set forth in SEQ ID NO: 57.  
15
10. The recombination vector of claim 3, wherein the Ig gene segment from a non-human animal is a gene segment coding for a heavy chain or light chain variable region.
- 20 11. The recombination vector of claim 10, wherein said vector is useful for replacing a rabbit heavy chain variable region gene and comprises a nucleotide sequence as set forth in SEQ ID NO: 52.
- 25 12. The recombination vector of claim 10, wherein said vector is useful for replacing a rabbit light chain variable region gene and comprises a nucleotide sequence as set forth in SEQ ID NO: 54.
- 30 13. A transgenic vector comprising a humanized Ig locus, wherein said humanized Ig locus is derived from an Ig locus or a portion of an Ig locus of a non-human animal and comprises multiple Ig gene segments wherein at least one of said gene

segments is a human Ig gene segment, wherein said gene segments are juxtaposed in an unrearranged, partially rearranged or fully rearranged configuration, and wherein said humanized Ig locus is capable of undergoing gene conversion and producing a repertoire of humanized immunoglobulins in said non-human animal.

5

14. The transgenic vector of claim 13, wherein said non-human animal is an animal which generates antibody diversity substantially by gene conversion.

15. The transgenic vector of claim 14, wherein said non-human animal is rabbit, pig, chicken, sheep or cow.

10

16. The transgenic vector of claim 13, wherein said humanized Ig locus is a heavy chain locus and comprises at least one V gene segment, at least one D gene segment, at least one J gene segment and at least one constant region gene segment.

15

17. The transgenic vector of claim 16, wherein said constant region gene segment is a human heavy chain constant region gene segment.

18. The transgenic vector of claim 17, wherein said human heavy chain constant region gene segment is a Cy.

20

19. The transgenic vector of claim 17, comprising about 10-100 V gene segments and at least one human V gene segment, wherein said human V gene segment is placed downstream to said 10-100 V gene segments.

25

20. The transgenic vector of claim 19, wherein said V gene segments are selected from V gene segments at the 3' V-region of said non-human animal and human V gene segments.

30

21. The transgenic vector of claim 13, wherein said humanized Ig locus is a light chain locus and comprises at least one V gene segment, at least one J gene segment and at least one constant region gene segment.
- 5 22. The transgenic vector of claim 21, wherein said constant region gene segment is a human light chain constant region gene segment.
23. The transgenic vector of claim 22, wherein said human light chain constant region gene segment is C $\lambda$  or C $\kappa$ .
- 10 24. The transgenic vector of claim 22, comprising about 10-100 V gene segments and at least one human V gene segment, wherein said human V gene segment is placed downstream to said 10-100 V gene segments.
- 15 25. The transgenic vector of claim 24, wherein said V gene segments are selected from V gene segments at the 3' V-region of said non-human animal and human V gene segments.
26. The transgenic vector of claim 22, wherein said human V gene segment is placed immediately 5' to a J gene segment in a rearranged configuration.
- 20 27. A method of making a transgenic vector comprising a humanized Ig locus capable of producing a functional repertoire of humanized antibodies in a non-human animal, comprising:
- 25 (i) obtaining a DNA fragment comprising an Ig locus or a portion thereof from said non-human animal which comprises at least one V gene segment, at least one J gene segment and at least one constant region gene segment; and
- (ii) integrating at least one human Ig gene segment into said DNA fragment of step (i) to produce a humanized Ig locus, wherein said human Ig
- 30

gene segment is linked to the sequences of non-human origin operably as to permit gene rearrangement and gene conversion of said humanized Ig locus and the production of a functional repertoire of humanized antibodies in said non-human animal.

5

28. The method of claim 27, wherein the integration of said human Ig gene segment is achieved by homologous recombination, thereby replacing an Ig gene segment in said Ig locus or said portion thereof from said non-human animal.

10

29. The method of claim 28, wherein the homologous recombination is achieved in a bacterial cell, a yeast cell, or a non-human animal cell.

15

30. The method of claim 28, wherein the human Ig gene segment is provided on a recombination vector, and is linked to a 5' nucleotide sequence and a 3' nucleotide sequence which are homologous to the 5' and 3' flanking sequences of said Ig gene segment from the non-human animal.

20

31. A transgenic animal comprising a humanized Ig locus, wherein said humanized Ig locus is derived from an Ig locus or a portion of an Ig locus of a non-human animal and comprises multiple Ig gene segments wherein at least one of said gene segments is a human Ig gene segment, said gene segments being juxtaposed in an unrearranged, partially rearranged or fully rearranged configuration, and wherein said humanized Ig locus is capable of undergoing gene conversion and producing a repertoire of humanized immunoglobulins in said non-human animal.

25

32. The transgenic animal of claim 31, wherein said animal is selected from rabbit, pig, chicken, sheep or cow.

30

33. A B cell from the transgenic animal of claim 31.

34. A method of making a transgenic non-human animal capable of producing a functional repertoire of humanized Ig heavy chains, comprising:
- (i) introducing a transgenic construct according to any one of claims 16-20 into a recipient cell of a non-human animal and integrating the humanized heavy chain locus in the transgenic construct into the genome of said recipient cell; and
  - (ii) deriving an animal from the recipient cell having the humanized heavy chain locus integrated in the genome, thereby producing a functional repertoire of humanized Ig heavy chains.
35. The method of claim 34, wherein said animal is rabbit and said recipient cell is a cell in an early embryo.
36. The method of claim 35, wherein said rabbit has an impaired expression of endogenous Ig molecules.
37. The method of claim 34, wherein said animal is chicken and said recipient cell is a fertilized egg.
38. The method of claim 37, wherein said chicken has an impaired expression of endogenous Ig molecules.
39. A method of making a transgenic non-human animal capable of producing a functional repertoire of humanized Ig light chains, comprising:
- (i) introducing a transgenic construct according to any one of claims 21-26 into a recipient cell of a non-human animal and integrating the humanized light chain locus in the transgenic construct into the genome of said non-human animal; and

- (ii) deriving an animal from the recipient cell having the humanized light locus integrated in the genome, thereby producing a functional repertoire of humanized Ig light chains.

5        40. The method of claim 39, wherein said animal is rabbit and said recipient cell is a cell in an early embryo.

41. The method of claim 40, wherein said rabbit has an impaired expression of endogenous Ig molecules.

10

42. The method of claim 39, wherein said animal is chicken and said recipient cell is a fertilized egg.

15

43. The method of claim 42, wherein said chicken has an impaired expression of endogenous Ig molecules.

44. A method of making a transgenic non-human animal capable of producing a functional repertoire of humanized antibodies, comprising:

- (i) introducing a transgenic construct according to any one of claims 16-20  
20        and a transgenic construct according to any one of claims 21-26 into a recipient cell of a non-human animal, and integrating the humanized Ig loci in the transgenes into the genome of said non-human animal; and
- (ii) deriving an animal from the recipient cell having the humanized Ig loci integrated in the genome, thereby producing a functional repertoire of  
25        humanized antibodies.

45. A method of making a transgenic non-human animal capable of producing a functional repertoire of humanized antibodies, comprising

- (i) making a transgenic non-human animal capable of producing a  
30        functional repertoire of humanized heavy chains;

- (ii) making a transgenic non-human animal capable of producing a functional repertoire of humanized light chains; and
- (iii) mating the transgenic non-human animal of (i) with the transgenic animal of (ii); and
- 5 (iv) selecting an offspring which produces both humanized heavy chains and humanized light chains thereby obtaining a transgenic non-human animal capable of producing a functional repertoire of humanized antibodies.

10 46. A humanized immunoglobulin produced using the transgenic animal of claim 31.

47. A humanized immunoglobulin derived from a transgenic animal, comprising at least a portion of a human immunoglobulin polypeptide sequence.

15 48. The humanized immunoglobulin of claim 47, wherein said transgenic animal generates antibody diversity by gene conversion and/or hypermutation

49. The humanized immunoglobulin of claim 48, wherein said transgenic animal is a rabbit, chicken, sheep or cow.

20 50. The humanized immunoglobulin of claim 49, wherein said human immunoglobulin polypeptide sequence is a heavy chain or light chain polypeptide sequence.

25 51. The humanized immunoglobulin of claim 50, wherein said portion of a human immunoglobulin polypeptide sequence is a human constant region polypeptide sequence.

52. The humanized immunoglobulin of claim 51, wherein said human constant region polypeptide sequence is C $\gamma$ , C $\kappa$ , or C $\lambda$ .

30

53. The humanized immunoglobulin of claim 51, wherein said portion of a human immunoglobulin polypeptide sequence further comprising a human V domain polypeptide sequence.
- 5      54. The humanized immunoglobulin of claim 47, wherein said humanized immunoglobulin is specific for an antigen.
55. The humanized immunoglobulin of claim 54, wherein said antigen is a microorganism selected from bacterium, fungus, or virus; an antigenic portion of said organism; an antigenic molecule derived from said microorganism; or a tumor-associated antigen.
- 10      56. The humanized immunoglobulin of claim 55, wherein said bacterium is selected from *S. aureus*, *Pseudomonas aeruginosa*, enterococcus, enterobacter, or *Klebsiella pneumoniae*.
- 15      57. The humanized immunoglobulin of claim 55, wherein said fungus is selected from *Candida albicans*, *Candida parapsilosis*, *Candida tropicalis*, or *Cryptococcus neoformans*.
- 20      58. The humanized immunoglobulin of claim 55, wherein said virus is selected from respiratory syncytial virus (RSV), Hepatitis C virus (HCV), Hepatitis B virus (HBV), cytomegalovirus (CMV), EBV, or HSV.
- 25      59. The humanized immunoglobulin of claim 55, wherein said antigen is selected from Her-2-neu antigen, CD20, CD22, CD53, prostate specific membrane antigen (PMSA), or 17-1A molecule.
- 30      60. An antibody preparation, comprising the humanized immunoglobulin of any one of claims 46-55.

61. The antibody preparation of claim 60, wherein said preparation is a monoclonal antibody preparation.
- 5 62. The antibody preparation of claim 60, wherein said preparation is a polyclonal antibody preparation.
63. The antibody preparation of claim 62, wherein said preparation is substantially non-immunogenic to human.
- 10 64. A pharmaceutical composition, comprising a pharmaceutically acceptable carrier and the antibody preparation of claim 60.
65. A method of treating a disease in a human subject comprising administering to  
15 said subject a therapeutically effective amount of the antibody preparation of claim 60.
66. The method of claim 59, wherein said disease is caused by bacterial, fungal or viral infection, or said disease is a cancer.

20

Figure 1(a)-(d). Novel nucleotide sequences 3'prime of the cow Cgamma gene (Cow Cy 3' flanking sequences). Primers are shown in shaded boxes. The 5' primer is in CH3, and the 3' primer is in M1. The sequences of clone 11, clone 3, and clone 5 are set forth in SEQ ID NO: 3, SEQ ID NO: 4 and SEQ ID NO: 5, respectively.

	1	50
clone11	CTTAC AGCTGCTGG TGTGCACGA AACTTTACGG	
clone3	CTTAC AGCTGCTGG TGTGCACGA GGCCCTGCAC	
clone5	CTTAC AGCTGCTGG TGTGCACGA GGCCCTGCAC	
	151	200
clone11	AATCACTACA AAGAGAAGTC CACCTCGAGG TCTCCGGGTA AATGAGCCTC	
clone3	AATCACTACA CGCAGAAGTC CACCTCTAAG TCTCGGGTA AATGAGCCTC	
clone5	AATCACTACA CGCAGAAGTC CACCTCTAAG TCTCGGGTA AATGAGCCTC	
	201	250
clone11	GCGCCGCTGA TCTAGTGGAC GTTCCCTCAT CCACCCACCC CTCCCCCACC	
clone3	ACGTCCCTGC ACCAGCAAGC CCTCACCAG C..... ..CCACCCTC	
clone5	ACGTCCCTGC ACCAGCAAGC CCTCACCAG C..... ..CCACCCTC	
	251	300
clone11	CCCGGGCTCC AGGTCCAGCC AGGGCGCCCT AGCCCTCCC TGTGTGCATT	
clone3	CCCGGGCTCC AAGTCCAGCC AGGACGCCCT AGCCCTCCC TGTGTGCATT	
clone5	CCCGGGCTCC AGGTCCAGCC AGGACGCCCT AGCCCTCCC TGTGTGCATT	
	301	350
clone11	CCTCCTGGGC CGCCGTGAAT AAAGCACCCA GGCCGCCCTG GGACCCTGCA	
clone3	CCTCCTGGGC CGCCGTGAAT AAAGCACCCA GGCCGCCCTG GGACCCTGCA	
clone5	CCTCCTGGGC CGCCGTGAAT AAAGCACCCA GGCCGCCCTG GGACCCTGCA	
	351	400
clone11	ACGCTGTGCT GGTTCCTTCC GAGGCAGAGC CCTGGTGGCC GCCAGGCCTG	
clone3	ACGCTGTGCT GGTTCCTTCC GAGGCAGAGC CCTGGTGGCC GCCAGGCCTG	
clone5	ACGCTGTGCT GGTTCCTTCC GAGGCAGAGC CCTGGTGGCC GCCAGGCCTG	
	401	450
clone11	CGGGGGTGGG CTGAGCCGAC TCTGGGCCAC TTTGTTTACG ATCTGTGGGG	
clone3	CAGGGGTGGG CTGAGCCGAC TCTGGGCCAC TTTGTTTACG ATCTGTGGGG	
clone5	CGGGGGTGGG CTGAGCCGAC TCTGGGCCAC TTTGTTTACG ATCTGTGGGG	
	451	500
clone11	GAGCTGACCC CACTCCGGGC CAGACACACA GTGAGTGGGT CCAGCAGGCC	
clone3	GAGCTGACCC CGCTCCGGGC CAGACACACA GTGAGTGGGT CCAGCAGGCC	
clone5	GAGCTGACCC CACTCCGGGC CAGACACACA GTGAGTGGGT CCAGCAGGCC	
	501	550
clone11	ACCTGGGGGC TGCCCAAGGC CACAGAGGGG CTTGGCCAGA GGCACAGCTC	
clone3	ACCTGGGGGC TGCCCAAGGC CACAGAGGGG CTTGGCCAGA GGCAGTACCTC	
clone5	ACCTGGGGGC TGCCCAAGGC CACAGAGGGG CTTGGCCAGA GGCACAGCTC	

Figure 1(a)

	551		600
clone11	CACGGTCCCC	TCCAGCCACC	ACCTGCTGGG CCGGCCTCTG GACAGGAACC
clone3	CACGGCCCCC	TCCAGCCACC	ACCTGCTGGG CCGGCCTCTG GACAGGAACC
clone5	CACGGTCCCC	TCCAGCCACC	ACCTGCTGGG CCGGCCTCTG GACAGGAACC
	601		650
clone11	GGGGAAGCCC	CCGAGACCCT	CAGGGATTGA GGCCCAATGC TTCCCGCCTC
clone3	GGGGAAGCCC	CCGAGACCCT	CAGGGATTGA GGCCCAATGC TTCCCGCCTC
clone5	GGGGAAGCCC	CCGAGACCCT	CAGGGATTGA GGCCCAATGC TTCCCGCCTC
	651		700
clone11	TGCTCCAGCC	CACGCTGTGG	GGCAGGGCCA CATCCTTGTC CCCAGGCCCC
clone3	TGCTCCAGCC	CACGCTGTGG	GGCAGGGCCA CATCCTTGTC CCCAGGCCCC
clone5	TGCTCCAGCC	CACGCTGTGG	GGCAGGGCCA CATCCTTGTC CCCAGGCCCC
	701		750
clone11	TGTCCTTGGG	TGTCCAGAGT	CCTTGTGTCC ACTCTGGGCC TGCTGGAGC
clone3	TGTCCTTGGG	TGTCCAGAGT	CCTTGTGTCC ACTCTGGGCC TGCTGGAGC
clone5	TGTCCTTGGG	TGTCCAGAGT	CCTTGTGTCC ACTCTGGGCC TGCTGGAGC
	751		800
clone11	CACGCATGGC	CAGGGGGTGG	CCCTGCTTCA CCCTCAGGCT CCCAAGGTCA
clone3	CACGCGTGGC	CGGGGGATGG	CCCTGCTTCA CCCTCAGGCT CCCAAGGTCA
clone5	CACGCATGGC	CAGGGGGTGG	CCCTGCTTCA CCCTCAGGCT CCCAAGGTCA
	801		850
clone11	GGCCTCGCCC	TCCCTCGGCC	AGGAGGCTCT GCCCGGCTCT CCCTGCCCAG
clone3	GGCCTCGCCC	TCCCTCAGCC	AGGAGGCTCT GCCCGGCTCT CCCTGCCCAG
clone5	GGCCTCGCCC	TCCCTCGGCC	AGGAGGCTCT GCCCGGCTCT CCCTGCCCAG
	851		900
clone11	GGCCAGGCCT	GTGCGCCCAT	GGGGAGGTCA TCCCTGTGCC TGAAAGGGGT
clone3	GGCCAGGCCT	GTGCGCCCAT	GGGGAGGTCA TCCCTGTGCC TGAAAGGGGT
clone5	GGCCAGGCCT	GTGCGCCCAT	GGGGAGGTCA TCCCTGTGCC TGAAAGGGGT
	901		950
clone11	CCAGGCCGAG	AGCCCTGAAT	GTCCAGGGCA GGGACCTAGC TGCTCCCTGT
clone3	CCAGGCCGGG	AGCCCTGAAT	GTCCAGGGCA GGGACCTAGC TGCTCCCTGT
clone5	CCAGGCCGAG	AGCCCTGAAT	GTCCAGGGCA GGGACCTAGC TGCTCCCTGT
	951		1000
clone11	GGACACGGAG	CCCAGAGCCA	CAGACAACAA GCCCCAGCCC CGCACGCACA
clone3	AGACACGGAG	CCCAGAGCCA	CAGACAACAA GCCCCAGCCC CGCACGCACA
clone5	GGACACGGAG	CCCAGAGCCA	CAGACAACAA GCCCCAGCCC CGCACGCACA
	1001		1050
clone11	CGAGACAGCC	CGCACCAGC	CTCCTCCACA CGCACTCAGG TGTACATCCG
clone3	CAAGACAGCC	CGCACCAGC	CTCCTCCACA CGCACTCAGG TGTGCATCCG
clone5	CGAGACAGCC	CACACCCGC	CTCCTCCACA CGCACTCAGG TGTGCATCCG

Figure 1(b)

	1051				1100
clone11	CACATGAGCA	CACTTCACCC	CGTCACACCC	ACACACCTAC	ACACACTCAG
clone3	CACATGAGCA	CACTTCACCC	CGTCACACCC	ACACGCCTAC	ACACACTCAG
clone5	CACATGAGCA	CACTTCACCC	CATCACACCC	ACACGCCTAC	ACACACTCAG
	1101				1150
clone11	GTCTCGCACT	CGGGGACCCA	TGGGGTGACC	CCACGGGCCC	AGA.CCAGAG
clone3	GTCTCGCACT	CGGGGACCCA	TGGGGTGACC	CCACAGGCCC	AGACCCAGAG
clone5	GTCTCGCACT	CGGGGACCCA	TGGGGTGACC	CCACAGGCCC	AGACCCAGAG
	1151				1200
clone11	CTGGGTCTTG	TGAGCCCTCC	CTGTGGACAC	CAGCTGGGCC	CCACCCCTCCA
clone3	CTGGGTCTTG	TGAGCCCTCC	CTGTGGACAC	CAGCTGGTCC	CCACCCCTCCA
clone5	CTGGGTCTTG	TGAGCCCTCC	CTGTGGACAC	CAGCTGGTCC	CCACCCCTCCA
	1201				1250
clone11	GCGCCCATGG	GCTGCTCAGC	GGCCCTTTCC	CACACTGACC	AACTGACCA
clone3	GCGCCCATGG	GCTGCTCAGT	GGCCCTTTCC	CACACTGACC	AACTGACCA
clone5	GCGCCCGTGG	GCTGCTCAGC	GGTCCTTTCC	CACACTGACC	AACTGACCA
	1251				1300
clone11	GGTCAGACAT	CCGTTCTTG	CCTCCCCTGG	GACACCCACG	CCCCTCCCTA
clone3	GGTCAGACAT	CCGTTCTTG	CCTCCCCTGG	GGCACCCACG	CCCCTCCCTA
clone5	GGTCAGACAT	CCGTTCTTG	CCTCCCCTGG	GGCACCCATG	CCCCTCCCTA
	1301				1350
clone11	GCAGGCTGAG	ATCCCCCCTC	AGCCCCTCGT	CCTGGCAGCC	TCACCCCTCG
clone3	GCAGGCTGAG	ATCCCCCCTC	AGCCCCTCGT	CCTGGCAGCC	TCACCCCTCA
clone5	GCAGGCTGAG	ATCCCCCCTC	AGCCCCTCGT	CCTGGCAGCC	TCACCCCTCA
	1351				1400
clone11	GGCACAGCAC	CCCTCAGGCC	CGGTGCTGTC	AGCCCTCCCT	CCCCGGGGGC
clone3	GGCACAGGGA	CAC...AGCC	CGGCGCTGTC	TGCCCTCCCT	CCCTGGGGGC
clone5	GGCACAGGGA	CAC...AGCC	CGGTGCTGTC	TGCCCTCCCT	CCCTGGGGGC
	1401				1450
clone11	AGGGCCCAGG	AACGTGCGCT	CTGCTGACCC	TCCCAGCTCC	AGGCCTGGCC
clone3	AGGGCCCAGG	CTCACATGCT	CTGCTGACCC	TCCCGGCTCC	AGGCCTGGCC
clone5	AGGGCCCAGG	CTCACATGCT	CTGCTGACCC	TCCCAGCTCC	AGGCCTGGCC
	1451				1500
clone11	CCCAGGGCAG	AGGAGGCCAG	GAAGTGAGCC	TCTGTCCTGT	GGGGAGGTAG
clone3	CCCAGGGCAG	AGGAGGCCAG	GAAGTGAGCC	TCTGTCCTGG	GGGGAGGTGG
clone5	CCCAGGGCAG	AGGAGGCCAG	GAAGTGAGCC	TCTGTCCTGG	GGGGAGGTGG
	1501				1550
clone11	GGTCAGGGTC	CCAGCTCAGG	GCACAGCTCA	GGATGGGAGC	AGGACCCAC
clone3	GGTCAGGGCC	CCAGCTCAGG	GCACAGCTCA	GGATGGGAAC	AGGACACCAC
clone5	GGTCAGGGCC	CCAGCTCAGG	GCACAGCTCA	GGATGGGAGC	AGGACACCAC

Figure 1(c)

	1551		1600
clone11	AGGCCAGGCC CAGATAGCAG CCAGGGCTGG AGGGGTGGG GCTGGGGCTG		
clone3	AGGCCAGGCC CAGACAGTGG CCAGGGCTGG AGGGGTGGG TCTGGGGCTG		
clone5	AGGCCAGGCC CAGACAGTGG CCAGGGCTGG AGGGGTGGG TCTGGGGCTG		
	1601		1650
clone11	GGCCCCAGAG ACTGACCTCA GGTGACCCCT GCCTGGCCCA TGGGGAGATC		
clone3	GGCCCCAGAG ACTGACCTCA GGTGATCCCT GCCAGCCCA TGGGGGATC		
clone5	GGCCCCAGAG AATGACCTCA GGTGATCCCT GCCAGCCCA TGGGGGATC		
	1651		1700
clone11	ACGCCACCTT CCCCCACCC AGAGGGAGCC CTGCCC...T ACCCCAGTGA		
clone3	CTGCCACCTT CCCCCACCC AGAGGGAGCC CTGCCCCGAG GCCCTGATGA		
clone5	CTGCCACCTT CCCCCACCC AGAGGGAGCC CTGCCCCGAG GCCCTGATGA		
	1701		1750
clone11	CCCTGCCCAG CCCTCCGTGG GCAGACACAG CACTGACCAC CCCTCCCTGT		
clone3	TGCCACCCAG CCCCCCGTGG GCAGACACAG CACTGACCAC CCCTCCCTGT		
clone5	TGCCACCCAG CCCCCCGTGG GCAGACACAG CACTGACCAC CCCTCCCTGT		
	1751		1800
clone11	GCAGACTTGC TGCTGGAGGA GGAGATCTGT GCGGACGACC TGGATGGGGA		
clone3	GCAGACCTGC TGCTGGAGGA GGAGATCTGT GCGGACGCC AGGACGGGGA		
clone5	GCAGACCTGC TGCTGGAGGA GGAGATCTGT GCGGACGCC AGGACGGGGA		
	1801		1850
clone11	GCTGGACGGG CTCGAGGCA CCAATGCGA CTT		
clone3	GCTGGACGGG CTCGAGGCA CCAATGCGA CTT		
clone5	GCTGGACGGG CTCGAGGCA CCAATGCGA CTT		

Figure 1(d)

Figure 2(a)-(e). Novel nucleotide sequences 3'prime of the sheep Cgamma genes. Primers are shown in shaded boxes. The 5' primer is in CH3, and the 3' primer is in M2. The sequences of clone 11 and clone 1 are set forth in SEQ ID NO: 8 and SEQ ID NO: 9, respectively.

	101		150
clone11	<b>CACGAGGCTC</b> TACACAACCA CTACACACAG	CACGAGGCTC	TACACAACCA CTACACACAG
clone1	<b>CCTAGGCTC</b> TACACAACCA CTACACACAG	CACGAGGCTC	TGCACAACCA CTACACACAG
	151		200
clone11	AAGTCGATCT CTAAGCCTCC GGGTAAATGA GCCACATGCC CCCGCACCAG		
clone1	AAGTCGATCT CTAAGCCTCC GGGTAAATGA GCCACACGCC CCCGCACCAG		
	201		250
clone11	CAAGCCCTCA CCCAGCCCGC CCTCCCCGGG CTCCAGGTCC AGCCAGGACG		
clone1	CAAGCCCTCA CCCAGCCCGC CCTCCCCGGG CTCCAGGTCC AGCCAGGACG		
	251		300
clone11	CCCTAGCCCC TCCCTGTGTG CATGCCTCCT GGGCCGCCAT GAATAAAGCA		
clone1	CCCTAGCCCC TCCCTGTGTG CATGCCTCCT GGGCCGCCAT GAATAAAGCA		
	301		350
clone11	CCCAGGCCGC CCTGGGACCC TGCAACGCTG TGCTTGTCTT TTCCGAGGCA		
clone1	CCCAGGCCGC CCTGGGACCC TGCAACGCTG TGCTTGTCTT TTCCGAGGCA		
	351		400
clone11	GAGCCCTGGT GACCGCCAGG CCTGCGGGGG GTGGGCTGAG CCCACTCTGG		
clone1	GAGCCCTGGT GATCGCCAGG CCTGCGGGGG GCGGGCTGAG CCCACTCTGG		
	401		450
clone11	GCCGCTTGGT TCAGCATCTG TGGGGGCGCT GACCCCTCTC CGGGCCAGAC		
clone1	GCCGCTTGGT TCAGCATCTG TGGGGGCGCT GACCCCTCTC CGGGCCAGAC		
	451		500
clone11	ACACAGTGAG TGGGTCCGGC AGGGCACCTG GGGGCTGCCC GAGGCCTCGG		
clone1	ACACAGTGAG TGGGTCCGGC AGGGCACCTG GGGGCTGCCC GAGGCCTCGG		
	501		550
clone11	AGGGGCTTGG CCAGAGGCGC AGCTTCACGG CCCCCTCCAG CCACCACATT		
clone1	AGGGGCTTGG CCAGAGGCGC AGCTTCACGG CCCCCTCCAG CCACCACATT		
	551		600
clone11	CTGGGCCAGA CTCTGGGCAG GAACGGGGGA AGCCCCGAC ACCTCAGGGA		
clone1	CTGGGCCAGA CTCTGGGCAG GAACGGGGGA AGCCCCGAC ACCTCAGGGA		
	601		650
clone11	TTGAGGCCCA ACGCTTCCCG CCTCTGCTCC AGCCCACGCT GAGGGGCAGG		
clone1	TTGAGGCCCA ACGCTTCCCG CCTCTGCTCC AGCCCACGCT GAGGGGCAGG		

Figure 2(a)

	651		700
clone11	GCCGCGGCCT TGTCCCCAGG CCCCTGTTCC TGGGTGCCCCA GAGTCCGTGT		
clone1	GCCGCGGCCT TGTCCCCAGG CCCCTGTTCC TGGGTGCCCCA GAGTCCGTGT		
	701		750
clone11	GTCCACTCTG GGCCTGCCTG GAGCCAGACT GGCCAGGGG GAGGCCCTGC		
clone1	GTCCACTCTG GGCCTGCCTG GAGCCAGACT GGCCAGGGG GAGGCCCTGC		
	751		800
clone11	TTCACCTCA GGCTCCCGAG GTCAGGCATC ATCTCGTCG GCCAGTAGCT		
clone1	TTCACCTCA GGCTCCCGAG GTCAGGCATC ATCTCGTCG GCCAGTAGCT		
	801		850
clone11	CTGCCTGGCT CTCTCTGCCC GGGGCCAAGC CTGTGTGCCC ATGGGGAGGT		
clone1	CTGCCTGGCT CTCTCTGCCC GGGGCCAAGC CTGTGTGCCC ATGGGGAGGT		
	851		900
clone11	CGTCCCTGTG CCTGAAAAGG GCCCAGGCTG GGAGCCCTGA ACGTCCAGGG		
clone1	CGTCCCTGTG CCTGAAAAGG GCCCAGGCTG GGAGCCCTGA ACGTCCAGGG		
	901		950
clone11	CAGGGACCTA GCTGCTCCCT GGGGACACTG AGCCAGAGC CCCAGACACC		
clone1	CAGGGACCTA GCTGCTCCCT GGGGACACTG AGCCAGAGC CCCAGACACC		
	951		1000
clone11	AAGCCCCAGC CCCGCACGCA CACGAGACAG CCCACACCCA GCGTCCTCCA		
clone1	AAGCCCCAGC CCCGCACGCA CACGAGACAG CCCACACCCA GCGTCCTCCA		
	1001		1050
clone11	CACGCACTCA GCGTCCACC CGCACACAAG CATGCTTCAC CCCCGTCACA		
clone1	CACCACTCA GCGTCCACC CGCACACAAG CATGCTTCAC CCCCGTCACA		
	1051		1100
clone11	CACCCACATG CCTGCACACA CTCAGGTCTC ACGCTCCGGG ACCCATGGAG		
clone1	CACCCACATG CCTGCACACA CTCAGGTCTC ACGCTCCGGG ACCCATGGAG		
	1101		1150
clone11	TGATCCCACG GGCCCAGACC CAGAGCTGGG TCTCATGAGC CCTCCCTGTG		
clone1	TGATCCCACG GGCCCAGACC CAGAGCTGGG TCTCATGAGC CCTCCCTGTG		
	1151		1200
clone11	GACACCAGCT GGTCCCCATT CTCCAGCGCC CTTGGGCTGC TCAAGTGGCC		
clone1	GACACCAGCT GGTCCCCATC CTCCAGCGCC CTTGGGCTGC TCAAGTGGCC		
	1201		1250
clone11	TTTCCACAC TGACCACACT GACCAGGTCA GACATCCTC CTCGCCTCC		
clone1	TTTCCACAC TGACCACACT GACCAGGTCA GACATCCTC CTCGCCTCC		

Figure 2(b)

	1251		1300
clone11	CTGGGGCACC CACGCCCCCTC CCTCGCAGGC TGAGACCCCC CCTCAGCCCC		
clone1	CTGGGGCACC CACGCCCCCTC CCTTGCAGGC TGAGACCCCC CCTCAGCCCC		
	1301		1350
clone11	TCGTCTCTGGC ACCCTCACCC CTCGGGCACA GGGACACAGC CCGGCACTGT		
clone1	TCGTCTCTGGC ACCCTCACCC CTCGGGCACA GGGACACAGC CCGGCACTGT		
	1351		1400
clone11	CTGCCCTCCC TCTCGGGGAC AGAGCCCAGG CACGTGTGCT CTGCTGAGCC		
clone1	CTGCCCTCCC TCTCGGGGAC AGAGCCCAGG CACGTGTGCT CTGCTGAGCC		
	1401		1450
clone11	TCCCGGCTCC AGGCCTGGCC CCCAGGGCAG AGGAGGCCAG GAATTGAGCC		
clone1	TCCCGGCTCC AGGCCTGGCC CCCAGGGCAG AGGAGGCCAG GAATTGAGCC		
	1451		1500
clone11	TCTGTCTCTGC GGGGAGGTGG GGTCAAGGCC CCAGCTCAGG GCACAGCTCA		
clone1	TCTGTCTCTGC GGGGAGGTGG GGTCAAGGCC CCAGCTCAGG GCACAGCTCA		
	1501		1550
clone11	GGATGGGAGC AGGACCCAC AGGCCAGGCC CAGACAGTGG CCAGGGCTGG		
clone1	GGATGGGAGC AGGACCCAC AGGCCAGGCC CAGACAGTGG CCAGGGCTGG		
	1551		1600
clone11	GGCTGGGGCT GGGGCCCAGA GACTGACCTC AGGTGACCCC TGCCCGGCC		
clone1	GGCTGGGGCT GGGGCCCAGA GACTGACCTC AGGTGACCCC TGCCCGGCC		
	1601		1650
clone11	ATGGGGGATC ACACCGCCAT CCCCCCGCC GCAGAGGGAG CCCTGCCCG		
clone1	ATGGGGGATC ACACCGCCAT CCCCCCGCC GCAGAGGGAG CCCTGCCCG		
	1651		1700
clone11	AAGCCCCGAT GGCCCCGCC AGCCCCCGT GGGCAGACAC AGCACTGACC		
clone1	AAGCCCCGAT GGCCCCGCC AGCCCCCGT GGGCAGACAC AGCACTGACC		
	1701		1750
clone11	CCCCCTCCTG TGCAGATCTG CTGCTGGAGG AGGAGAGCTG TGCGGACGCC		
clone1	CCCCCTCCTG TGCAGATCTG CTGCTGGAGG AGGAGAGCTG TGCGGACGCC		
	1751		1800
clone11	CAGGACGGGG AGCTGGACGG GCTCTGGACG ACTATCTCCA TCTTCATCAC		
clone1	CAGGACGGGG AGCTGGACGG GCTCTGGACG ACTATCTCCA TCTTCATCAC		
	1801		1850
clone11	GCCCTTCCTG CTCAGCGTCT GCTACAGCGC CACCGTGACC CTCTTCAAGG		
clone1	GCTCTTCCTG CTCAGCGTCT GCTACAGTGC CACCGTGACC CTCTTCAAGG		

Figure 2(c)

	1851		1900
clone11	TGGGGGTCCA CCCTGCTGGG CCCTCGGGCC CCCTCTCTGT CCCCAGGGTC		
clone1	TGGGGGCCCA CCCTGCTGGG CCCTCGGGCC CCCTCTCTGT CCCCAGGGTC		
	1901		1950
clone11	CCCGCAGAGT CCCTCCCTGC CCCTCACTGT CCCTCCCTGT CCCTCTCTGT		
clone1	CCCGCAGAGT CCCTCCCTGC CCCTCACTGT CCCTCCCTGT CCCTCTCTGT		
	1951		2000
clone11	CCCTCTCTGT CCCTCTCTGT CCCTCTCTGT CCGTTCATTT TCCCTTCACC		
clone1	CCCTCTCTGT CCCTCTCTGT CCCTCTCTGT CCGCTCATTT TCCCTTCACC		
	2001		2050
clone11	GTAAGCTTGA GACAGATTGG GGTCAATTTCA GAGGGCGTCT GAAGAGTCTC		
clone1	GTAAGCTTGA GACAGATTGG GGTCAATTTCA GAGGGCGTCT GAAGAGTCTC		
	2051		2100
clone11	TGTGCCGCAC GCCTCCCTTC ATGTCAGTGG GGAGAATTCA GCAAGGGTGG		
clone1	TGTGCCGCAC GCCTCCCTTC ATGTCAGTGG GGAGAATTCA GCAAGGGTGG		
	2101		2150
clone11	AGTGCTGGGT GAGAAATGAG GCTTGCGGCG CTCACGAGCA GTGATGGGGC		
clone1	AGTGCTGGGT GAGAAATGAG GCTTGCGGCG CTCACGAGCA GTGATGGGGC		
	2151		2200
clone11	ACTGCTGCTC CCTGAGACCT GCGCGGACAC CGTTTTCCAT CGCAGGAGAA		
clone1	ACTGCTGCTC CCTGAGACCT GCGCGGACAC CGTTTTCCAT CGCAGGAGAA		
	2201		2250
clone11	GCGGGCAAGG GAAAACGCCC TCTTGGTCTC TCTTGAGTAA ATGTCGCGTT		
clone1	GCGGGCAAGG GAAAACGCCC TCTTGGTCTC TCTTGAGTAA ATGTCGCGTT		
	2251		2300
clone11	TTGGTCATCA GTCCCTCCCC CAGTGAGGCT AGAGGAGTTT ACTTCTCCCT		
clone1	TTGGTCATCA GTCCCTCCCC CAGTGAGGCT AGAGGAGTTT ACTTCTCCCT		
	2301		2350
clone11	CTCGATGGTC AGGTCAGGAC TGTCATAGAC TCCGGATCAC CTTCCGTGTA		
clone1	CTCGATGGTC AGGTCAGGAC TGTCATAGAC TCCGGATCAC CTTCCGTGTA		
	2351		2400
clone11	ATGCTTGCTT TTTGTGTGCA GAGAGCCTGT TTTAGCTCGG GGGTCCTCAG		
clone1	ATGCTTGCTT TTTGTGTGC .AGAGCCTGT TTTAGCTCGG GGGTCCTCAG		
	2401		2450
clone11	CTCACTGAGC TCGCGGGGCA GGGGTGGGCT CGGGCTGGCG CCGCCTGTTC		
clone1	CTCACTGAGC TCGCGGGGCA GGGGTGGGCT CGGGCTGGCG CCGCCTGTTC		

Figure 2(d)

	2451		2500
clone11	GGGAGCGCAT CTCCAGCATG CTGTCGCACA GCTTCGTTGC TAACAAGACC		
clone1	GGGAGCGGCA TCTCCAGCTG CTGTCGCACA GCTTCGTTGC TAACAAGACC		
	2501		2550
clone11	GCTTAGTCTC GTGGTTAGAC CAACCTGCTT TCTCGAGTAA TTGTTAATTT		
clone1	GCTTAGTCTC GTGGTTAGAC CAACCTGCTT TCTCGAGTAA TTGTTAATTT		
	2551		2600
clone11	ACAGGAGTTT CCTGTATTTT TCAACTTATA ATCCCCTAGT CAGATAACTC		
clone1	ACAGGAGTTT CCTGTATTTT TCAACTTATA ATCCCCTAGT CAGATAACTC		
	2601		2650
clone11	TTTAATCACC TATTCTGCCC CTTCAATTTTC TCCCTATCGA TCTCAGCAAC		
clone1	TTTAATCACC TATTCTGCCC CTTCAATTTTC TCCCTATCGA TCTCAGCAAC		
	2651		2700
clone11	CCATCACTGC CCTCACTGTC CTTAAACTGT CCCTTAACTG ACCAGACTGT		
clone1	CCATCACTGC CCTCACTGTC CTTAAACTGT CCCTTAACTG ACCAGACTGT		
	2701		2750
clone11	CCCTCAGTGT CCCCTCAGAG TCACCTCCCT ATCACCTCAC TGTCCCTCTC		
clone1	CCCTCAGTGT CCCCTCAGAG TCACCTCCCT ATCACCTCAC TGTCCCTCTC		
	2751		2800
clone11	TGCCCCCTCTC TGCCCCCTCTC TGTCCCTCCC TGCCCCCTCCC CGTCCCCTCT		
clone1	TGCCCCCTCTC TGCCCCCTCTC TGTCCCTCTC TGTCCCTCCC CGTCCCCTCT		
	2801		2850
clone11	CTGTCCCTCT CTGCCCCCTCA CTGCTCCTCT CTGCACCTCA CTGCTCCTCA		
clone1	CTGTCCCTCT CTGCCCCCTCA CTGCTCCTCT CTGCACCTCA CTGCTCCTCA		
	2851		2900
clone11	CTGCCCTGGG GGAGGCCCGC ATCGAGGTGT CTCTGCTCAC CCCGTCCCCC		
clone1	CTGCCCTGGG GGAGGCCCGC ATCGAGGTGT CTCTGCTCAC CCCGTCCCCC		
	2901		2950
clone11	ACCCCGTACC CCCCGCCAGG		
clone1	ACCCCGTACC CCCCGCCAGG		

Figure 2(e)

Figure 3(a)-(b). Novel 3'prime flanking sequence (SEQ ID NO: 10) of rabbit Cgamma gene.

TGTGCACCCCGCACACAAATAAAGCACCCAGCTCTGCCCTGAGAGGCTGTCTCTGATTCCCT  
TCCAAGGCAGAGGCTTCCACTCGGGCCGGACAGGGTTGGGCGGGCGCCGTGGGCTCTGCT  
GTGGCCAGCAGCCAGAACGGTCAACAGTGGGACAGGGGCAGACCCACAGCACAGGGGCT  
GCCAAGAAGTGGGCTCAGCCGGAGTGTGTGGCAGGTCCCCCTTGACGCTAGCACGTGT  
GTGCTGGGACAGGAGGGCCCCAGGGGAGGAGCACACAGCTACCACCTCTGCAAGAGCC  
TGGCCTGGGCGCCAGGTCCAGTCCACAGGGTGTGTAGTACACAGAGCCTCATCTTACCA  
CAGATGTAGGGACAGACCCACACGCCCCCTGCACCCACCCAGCCTCGCCCCCTTGTGGGA  
CCAGGGCTACCACTCCACTCCCCGCCCCAGAGCAGCAGAAGCAGGTGGCATCTCTCAGCAG  
AGGCACAGTCTCACCCCTCCACGGCACTGAGCCCTGACCCATCAAACAAGCCCCCTCTGCT  
TGCACAGCAGCTGTGTGCACATCACACACACACACACACACACTGAGGCCCTGACCCCA  
TCAAACAAGCCCCCTCTGCTGCACAGCACCTGTGTGCACATCACACACACACACACAC  
ACACTGAGGCCTGACCCCATCTGCCCCCTCTGCTGCATGGCACCTGTGTGCACATCACAC  
ACACATGCACACACACACTCACACACACTGAGCCCTGACCCCATCTGCCCCCTCTGCTGC  
ATGGCACCTGTGTGCACATCACACACACACACACACACACACACTGAGCCCTGACC  
CCATCTGCCCCCTCTGCTGCATGGCACCTGTGCACACATCACAAACAGCCCTGCCTCATA  
CACTGGCACTCAGAAGGGGCCCTGTACACGCATACACATGCACACACCTTGACACATGG  
GCCCCCTACACACGCATCACACACACTCATGCACACTCTCTCACACATGGCCCCCTCTGCAC  
ATACATTGCACACACATGTGCACAGACCTCACAAATGGGCCCCCTGCACACACATTGTACAC  
ACGCATGTGCACACACTTCACACATGGGCCCCCTGCACATGCATTGCACACACAGACACA  
CACATGTGCATTCTCTCACACATTGGGGCCTTGCAAGGGATGCCCTGCACACACATTGCAC  
ATGCTCACATGTGCACACACCCACACTGGAGCCTTGACATAGGGCCCCCTGTACACACAC  
CATGCATACACACACACCTCACACAGGGGGCCCCCTACATACGCAAAACACACACACACA  
CATGCACACACCTCATACAGGGGCCCTACACACATCACACACACACACACACACAGT  
ATGCATGCTCACACACAGACCTTGCAAGGGGGCCCCCTGCACATGCATCAAACACATATG  
CACATGTTTCACACACACGGTCCCCCTACACACACTGCACACGCACACATGTGTACATGCT  
TCACACACTGGGGCCTTGACATGGGGTCCCCCTGCATAGCATAGCACCCAGAGCCACGCCAG  
GTGCTTGGGCACATGGCACTGGTGCACACACAGCACCCCAAGCCAGCTCTCCCATCAA  
GGGGCACACACACCCCACTCACAGCACCCCTGAATTCTGCTCCCCACAGCGAACGT  
GCACCTTACCTCTCCAGACGTCCCTTTCTGTGGCCACTCCCATAGGTATTGGCGAGACC  
CTCCCTTGACCCTTGGGCTTGGTCAACCCAGGGGACAGGAGAGGGCCAAGTTGGGCCACAG  
TACCACTGCCCAGCAGGGGTGAGGCAAGCAGAGGGTGGGTCTGTGAGGCGTCTGGCCAGC  
CGTGTGCGGGCCAGGTGGGGAGCAGCTGGGTGGCTGAGGTGGCTTCTTGCAGGTGGTT  
GGGGGAGCTGGCCCCACAAGTGCCACTGCCAGCACTGTCCAGTGTCTCCCCCTGAACC  
TCCCGGCCACCATCCCCAGCTGCAGCCGAGAGGGAGTGCCCTCGGCCCTCTCGGCCAA  
GACGCACGCTGACTGCCCTCCCCATCCAGAGCTGCAGCTGGACGAGAGCTGTGCCGAGG  
CCCAGGACGGGGAGCTGGACGGGCTGTGGACCACCATCACCATCTTCATCTCCCTCTTCC  
TGCTCAGCGTGTGCTACAGCGCCACAGTCAACCTCTTCAAGGTGGGTGCTGCACCCGGCA  
CGGGTGGGCTGGGGGCCAGGGGCGGGGGCCGGGGCCAGGCCCTCTCACCCCCGCGCCG  
CGCTGCTGCAGGTGAAGTGGATCTTCTGCTCGTGGTGGAGCTGAAACACACCATCGCTC  
CCGACTACAGGAACATGATCGGGCAGGGGGCCCTAGGCCCTTCGTTCTCACAGCCTGCCTC  
CCTGGCCAGCAGGAGCCCCCGCCTCCGCCCTCGGACCCCATGGCTCTCTGCTCTGGCCGCT  
CCGGACCCCTCCGCCCTCGGGAGAAGCGCGCAGCTGATGCCCTGCCGGCCCCCTCCACGCAGC  
AGTGCGGACAGCACGCATCTGTCTCCACCCGGCAGGACCCACCCAGGGCCAGCCCTGA  
CCGCCAGCCTCTGGACTCAGGGCTCCTCTGAGAAAAGGCCCACTTGTTGGTCCCCCTCAG  
CCCACACCCAGGCAGCCTCCGGTGGGTGCTTCCCTGGACCCACGCCTGAGGCCTATGCTT  
GTTCTCTGTGGCTCTTACTCAGAGGCCCGTGTGGACTCCACCCACAGGGACAGTGCC

Figure 3(a)

CTGCTCCAACCCTCACTGCACTGGGGGTCATGGGGCCACCTTCTGTGCAGGGGTCTGGC  
TCCAAGGAGAACACTCGAAGGGCCTGCTTGGCCACCTGGCACCACGGGAGCCCCGCTGGG  
TAGCTTGGCAGGGACCCCTGAGTAGAGGTGGGTGCACCCAGCCAGAAAGCCTGCTGGATG  
GACAGGAGCCTGGCGTCCGGGCCCCAGGCAGGCAGACACGGCTTCATGGACAGGAGAGGC  
CAAGGAACATCAGCAAAGAGAGACAGCTGGGCCGGGCGTTCCAGCCAGACCCATCCTGCA  
GCCCAGCATCGGCC

Figure 3(b)

Figure 4. Novel nucleotide sequence (SEQ ID NO:11) 3'prime of the rabbit Ckappa 1 gene.

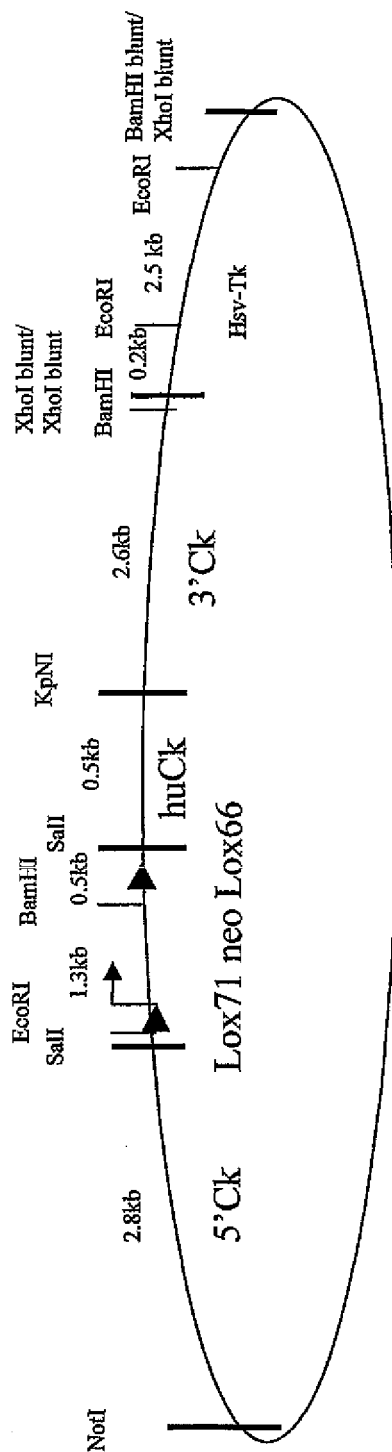
GGGATACGGCTGACAGGATGCTGAGTGACCCCTGAG  
GCCAGCCTCGCGGCTCCCTCCCTCAGTGGACCCATTCCACCACAGTCCTCCAGCCCC  
TCCCTCCCGGCCCTCACCCTCCTTGGCTTTAACCTTGGCAATGTTGGTGAGATGGAT  
GAATAAAGTGAATCTTTGCACTTGTGACTTCTCTCTGCTTCTTCATTTAATGGTTATTAC  
TCATGGTTTCCCAGTTGCCTAAAGTCACCGCCATTTTCATCCTCCATCCCACCCTGCCCT  
GCTGTCTCCGGGAGACACCACTCCCTGAAACCCACAGGCCCCCTGTCTTCACACCGCCGA  
CCCCGACCACACGTGAGGGCTTGCCTCGTGTCTCACTCCCTCATCGAGCCCCAGAGTC  
CTCCTTTAGTGTCTTACAGTCACATACAGTTATACAGTTTGAGTCAATCCAACTGCCCT  
TGCCAATTTCCAAAACAAAGATTTTCAGAATAAAACAGCTATGAAGAAAGTCATTTATG  
GAAGCATGATATACAACAACAAACAAATGCAAAACACCTAACTGAATAAGCAGAGGGAAA  
TGTTTCAGACACACTATGGGGCTTGGGCTTCATGGAGTATTACACCTTCATTACATTTTAA  
AACTTGATTAAGGAGCTCCTATATTACAAGGATTATACTAGAGCACTTTCCATGACCTA  
ATTAATTTCTATTACACTGTGAGGTTAAAAGCATTAGTTAAAATATTGGGCAGGCTCCCT  
ATAGCCACAGTTGTTTCATATTCCATAACCCCAACCATCATTTAGGTGACTCAGGGTCCTT  
GTCCACCAAGAACTTTGGCAAGAATGTTCAAGCAACTTCCTTTATAAAAGTCAAAAATT  
GGAAGTAACTCAAATGTCTACCAACAGTAGAATGGGCTGTTAATTGGCATATGTTTACAT  
ATTAGAATGCTGTTTAAATAAGAGAAATTAACAACACTACAATATCCCTAATAACATAGGT  
GACTCATAAACATGATGTTAAGCACAAGAACCCAAACACAAAAGACACACTGTGTATGTT  
TTCATCCATAGGAAGTTCAAAACACTAGTTAAAAATTGAATTAGAAATTGAGATGAAGTTA  
CTCTTGGCTGGGGGTGTGGAGTGAGCGGCTGCCTGGTGGGGGACAGAAAGTGGCTGCTGG  
GGTCTTGGTGATGTTCTAGTCCCTCACTGTGGTGTGTGCTACTCTGAAAATGTATTGAGTA  
CACAATTAGGTTTTGTGCTTTTCATTTATACTCCAAAGTAAGTTCTCATAAACATTGCCTTA  
CACGGGGTCTACAGATAAGAGAGACTAAGAGGAATGAGTAACAGATCAAGGCCACACAGC  
TGGTAGGCATGGGCTGGGATCAAACCTGTCTGCCCAATTCCTGCTCTCTTGAGCCCTAC  
ACTATTCTTCCAGCACTGGAATGCCATGCAGAACAGGGAGTAGGACATGCTACCTCCCT  
AGGGTCTCCTCCTTACCCACCTAACCCAGGAGCACCCATACATAGAAACAGGATGGAAAA  
GACCATCAGCAATGGAACAAGGGAGAGATTAACTTGTTCAGTATTGTGATCCCATGTAG  
GAAAGATTGTGGGAGGAGGGCTGCACACAGAGCACCGTCCCCCTTCTATGTGCCACCCGC  
TCTGTGCCCCCTTATCTGCTCACCCGCCCCAGCGTGCACTCACTCAGCACCCCTTTTCGCCT  
GCCCTCTGAAAGAGGTGCAGAAGTAACTAAACAGCTTCCTCCTTCAGTGACTTGAAT  
CCAGTTTTCTCCTCTATTTCCCCCTCCTTTTCAGTGCAGGAGCCTGGAGAAATGTGATT  
TGTGTTATTATAAATTTCCCATCATTTTGTGTAAGGGAAAAATATACTCAACAGTCATA  
ACTGGTAAAACTGCTGTGAAAACAAAGAGAAGTAATTCATGCGAAGGTTGAGCACCAGCC  
TTGTATATACTAAGAGATCCAGAAGTGTAGTCACCGTTAGAAATAAGAAGGAGTAGCTC  
AATTTGACTAGTTCCTGGTTCACTCCTTGAACATGTTCTTCAGTTATCATCTTTCAGTCC  
CAATGATTGAACTTGAATTAACACATGGATTCTAGACCTGTGCCGAGAATGGCTGC  
CACTCGTGCTCTAGAGCTCTGGGGATGAGGCTGTCCCTACTGTGGTGTGCTACAGGTCTA  
ACAACACACCAGGTTTTGAAGACTTAGCACTATGAATATATATATATATTATATATCCAA  
AAATTTAACATACTTTCTACTTTTCATTGCATGTTGAGATAGTAATCTACTTTGGATATAT  
TTGGTTAAACCAAACTATTCTCAAGACAAATTTCATAGGTTTATGGTTTTTTTACAATTT  
AATCAAAATATAAAACATAGTCCAAACAATTAATCCATTTAAAGTGGAGAATGGCCCAAGT  
GTTTGGGCCCCCTGCTACCCATTTTAAAGACCAGATGTTGCTCTTGGCTTCTGGCTTTTGC  
TTGGCTCAGCCCTGGCCATTGCAGCCATCTGAGGAGTAAACAGTGGATGGAAGACATCTC  
GGCCACCGCTCCGATTAAGGAGTGG



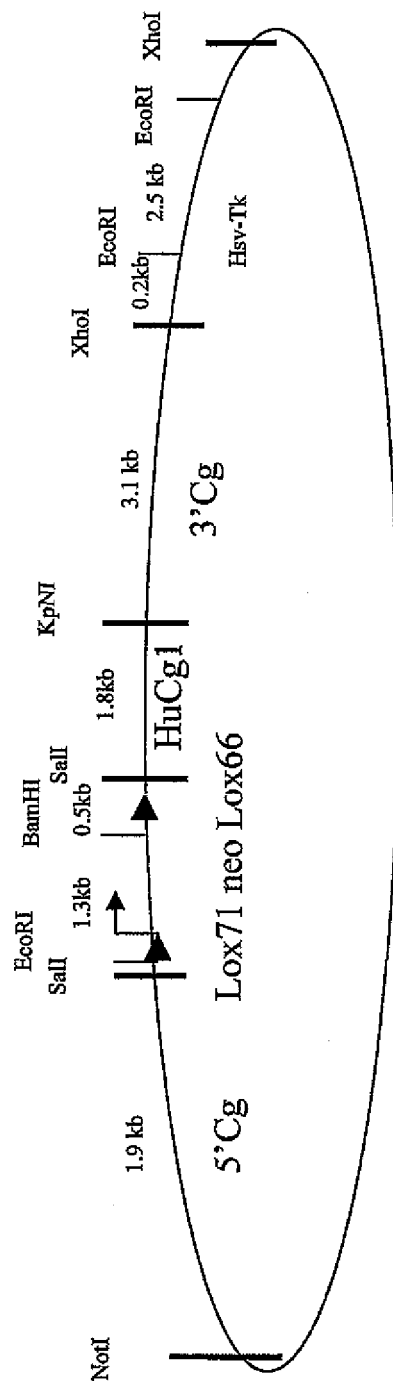
Figure 6. Comparison of human, mouse, rabbit, sheep, cow and camel sequences for the the M1 and M2 regions 3' of the Cgamma gene.

M1	1	46	SEQ
camel	EPLEEESCA EAQSGELDGL WTTISIFITL FLLSVCYSAT VTLEFK.	14	
human-Ig3	.ELQLEESCA EAQDGELDGL WTTITIFITL FLLSVCYSAT VTFFK.	15	
human-Ig3/2	.ELQLEESCA EAQDGELDGL WTTITILITL FLLSVCYSAT VTFFK.	16	
human-Ig1	.ELQLEESCA EAQDGELDGL WTTITIFITL FLLSVCYSAT VTFFK.	17	
mouse-Ig1	.GLQLDETCA EAQDGELDGL WTTITIFISL FLLSVCYSAA VTLEFK.	18	
mouse-Ig2a	.GLDLDDVCA EAQDGELDGL WTTITIFISL FLLSVCYSAS VTLEFK.	19	
mouse-mRNA	PGLQLDETCA EAQDGELDGL WTTITIFISL FLLSVCYSAA VTLEFK.	20	
mouse-Ig3	.ELELNETCA EAQDGELDGL WTTITIFISL FLLSVCYSAS VTLEFK.	21	
mouse-Ig3/2	.ELELNGTCA EAQDGELDGL WTTITIFISL FLLSVCYSAS VTLEFK.	22	
sheep-clone11	.LLEEESCA DAQDGELDGL WTTISIFITP FLLSVCYSAT VTLEFK.	23	
sheep-clone1	.LLEEESCA DAQDGELDGL WTTISIFITL FLLSVCYSAT VTLEFK.	24	
cow-clone11	.LLEEESCA DDLGELDGL .....	25	
cow-clone3/5	.LLEEESCA DAQDGELDGL .....	26	
rabbit	.LQLEESCA EAQDGELDGL WTTITIFISL FLLSVCYSAT VTLEFK.	27	

M2	1	27	SEQ
camel	VKWIFSSVVE LKRTIVPDYR NMIGQGS	28	
human-Ig3	VKWIFSSVVD LKQTIIPDYR NMIGQGA	29	
human-Ig3/2	VKWIFSSVVD LKQTIIPDYR NMIGQGA	30	
human-Ig1	VKWIFSSVVD LKQTIIPDYR NMIGQGA	31	
mouse-Ig1	VKWIFSSVVE LKQTLVPEYK NMIGQAP	32	
mouse-Ig2a	VKWIFSSVVE LKQTIIPDYR NMIGQGA	33	
mouse-mRNA	VKWIFSSVVE LKQTLVPEYK NMIGQAP	34	
mouse-Ig3	VKWIFSSVQ VKQTAIPDYR NMIGQGA	35	
mouse-Ig3/2	VKWIFSSVQ VKQTAIPDYR NMIGQGA	36	
rabbit	VKWIFSSVVE LKHTIAPDYR NMMGQGA	37	
sheep-clone1/11	VKWIFSSV.. .....	38	



**Figure 7a: DNA construct for the replacement of rabbit Cκ with human Cκ.** A 0.5 kb fragment containing a DNA sequence encoding human Cκ is flanked by sequences from the rabbit CκI gene. The upstream sequence (5'Cκ) is 2.8 kb, the downstream sequence (3'Cκ) is 2.6 kb. The vector also contains a lox-neo cassette for positive selection and a Hsv-Tk cassette for negative selection.



**Figure 7b: DNA construct for the replacement of rabbit  $C\gamma$  with human  $C\gamma$ .** A 1.8 kb fragment containing a DNA sequence encoding human  $C\gamma$  is flanked by sequences from the rabbit  $C\gamma$  gene. The upstream sequence (5'C $\gamma$ ) is 1.9 kb, the downstream sequence (3'C $\gamma$ ) is 3.1 kb. The vector also contains a lox-neo cassette for positive selection and a Hsv-Tk cassette for negative selection. The figure is not up to scale.

Figure 8. DNA fragment (SEQ ID NO: 51) containing a human immunoglobulin heavy chain C $\gamma$ 1 gene segment flanked by 50 nucleotides derived from the rabbit heavy chain immunoglobulin gene. Flanking sequences derived from rabbit immunoglobulin DNA sequences are underlined.

```

aagggatcac atggcaccac tgacctacct accctgccaa ggtcaggggt cctccaaggc
ctggcaccct cctccaagag cactctctggg ggcacagcgg cctggggctg cctgggtcaag
gactacttcc ccgaaccggg gacgggtgtcg tggaaactcag gcgccctgac cagcggcgtg
cacaccttcc cggtctgtcct acagtctctca ggactctact ccctcagcag cgtgggtgacc
gtgccctcca gcagcttggtg caccagagacc tacatctgca acgtgaatca caagcccagc
aacaccaagg tggacaagaa agttgggtgag aggccagcac agggagggag ggtgtctgct
ggaagccagg ctccagcgctc ctgcctggac gcctcccgcc tatgcagccc cagtccaggg
cagcaaggca ggccccgtct gctctctcac ccggaggcct ctgcccgccc cactcatgct
cagggagagg gtcttctggc tttttcccca ggctctgggc aggcacaggc taggtgcccc
taaccaggc cctgcacaca aaggggcagg tgctgggctc agacctgcca agagccatat
ccgggaggac cctgccccctg acctaaagccc accccaaagg ccaaactctc cactccctca
gctcggacac cttctctcct cccagattcc agtaactccc aatcttctct ctgcagagcc
caaattttgt gacaaaactc acacatgccc accgtgccca ggtaagccag ccagggcctc
gccctccagc tcaaggcggg acaggtgccc tagagtagcc tgcattccagg gacaggcccc
agccgggtgc tgacacgtcc acctccatct cttctcagc acctgaactc ctggggggac
cgtcagtcct cctcttcccc caaaaaccca aggacacct catgatctcc cggaccctg
aggtcacatg cgtgggtgtg gacgtgagcc acgaagaccc tgagggtcaag ttcaactggt
acgtggacgg cgtggagggtg cataatgcca agacaaagcc gcgggaggag cagtacaaca
gcacgtaccg tgtgtgtcagc gtctcaccg tctgtcacca ggactggctg aatggcaagg
agtacaagtg caaggtctcc aacaaagccc tcccagcccc catcgagaaa accatctcca
aagccaaagg tgggaccctg ggggtgcgag ggccacatgg acagaggccg gctcggccca
ccctctgccc tgagagtgac cgctgtacca acctctgtcc ctacagggca gccccgagaa
ccacagggtg acaccctgcc cccatcccgg gatgagctga ccaagaacca ggtcagcctg
acctgcctgg tcaaaggctt ctatcccagc gacatcgccg tggagtgga gagcaatggg
cagccggaga acaactacaa gaccacgcct cccgtgctgg actccgacgg ctcttcttc
ctctacagca agctcaccgt ggacaagagc aggtggcagc aggggaacgt cttctcatgc
tccgtgatgc atgaggctct gcacaaccac tacacgcaga agagcctctc cctgtctccg
ggtaaatgag cgctgtgccg gcgagctgcc cctctccctc cccccacgc cgcagctgt.

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Figure 9. The DNA fragment (SEQ ID NO: 52) containing a VH gene segment with more than 80% sequence identity with rabbit VH elements and encoding a human VH element polypeptide sequence. Flanking sequences derived from rabbit immunoglobulin DNA sequences are underlined.

tgagtgacag tgtcctgacc atgtcgtctg tgtttgcagg tgtccagtgt  
gaggtgcagc tgttggagtc cgggggaggt ctctccagc caggggggac cctgagactc  
acctgcgcag tctctggatt caccttcagt agctatgcaa tgagctgggt cggccaggct  
ccaggggaag ggctggaatg ggtcggagcc attagtggta gtggtagcac atactacgcg  
gacagcgtga aaggccgatt caccatctoc agagacaact ccaagaacac gctgtatctg  
caaatgaaca gtctgagagc cgaggacacg gccgcctatt actgtgcgaa agacacagtg  
aggggccctc aggctgagcc cagacacaaa cctccctgca

Figure 10. DNA fragment (SEQ ID NO: 53) containing a human immunoglobulin light chain Ck gene segment flanked by 50 nucleotides derived from the rabbit light chain immunoglobulin Kappal gene. Flanking sequences derived from rabbit immunoglobulin DNA sequences are underlined.

ggagatgtcc actggtacct aagcctcgcc atcctgtttg .catccacat ggcacccagg  
ggtgtcacca tctgtcttca tcttcccgcc atctgatgag cagttgaaat ctggaactgc  
ctctgtttgt tgcctgtga ataacttcta tcccagagag gccaaagtac agtggaaggt  
ggataacgcc ctccaatcgg gtaactccca ggagagtgtc acagagcagg acagcaagga  
cagcacctac agcctcagca gcaccctgac gctgagcaaa gcagactacg agaaacacaa  
agtctacgcc tgcgaagtca cccatcaggg cctgagctcg ccgtcacaa agagcttcaa  
caggggagag tgttagagcg agacgcctgc cagggcaccg ccagcgaccc tgaggccca  
cctcgc.

Figure 11. DNA fragment (SEQ ID NO: 54) containing a Vk gene segment with more than 80% sequence identity with rabbit Vk elements and encoding a human Vk element polypeptide sequence. Flanking sequences derived from rabbit immunoglobulin DNA sequences are underlined.

catgcaggag gcagtaccag gcaggaccca gcatggacat ..ggcaggct gctccaccc  
gaggggtccct gctcagctoc  
tgggactcct gctgctctgg ctcccaggta aggagggaaa caacaaaaat tttattcagc  
cagtgtagcc actaatgcct ggcaattcag gaaattcttc ttagaacatt actaatcatg  
tggatatgtg tttttatggt cctaatatca gataccagat gttacatcca gatgaccag  
tctccatcct ctctgtctgc atctgtggga gacagagtca ccatacttg ccgagccagt  
cagggcatta gcaattactt agcctggtat cagcagaaac caggggaaggt tcccaagctc  
ctgattttatg ctgcatccac tttgcaatct ggggtcccat ccgggttcag tggcagtggga  
tctgggacag atttactct taccatcagc agcctgcagc ctgaagatgt tgccacctat  
tactgtcaaa agtacaacag tgcccctcca cttttcggcg gagggaccaa ggtggagatc  
aaacgtaagt gcactttcct aatgttcctc accgtttctg cctgatttgt ttgctttttc  
cattttttcgtat..

Figure 12. DNA fragment (SEQ ID NO: 57) containing a gene encoding human immunoglobulin light chain constant region Clambda2 flanked by 50 nucleotides derived from the chicken light chain gene. The DNA sequence of chicken origin is underlined.

```
catacacag ccatacatat gcgtgtggcc gctctgcctc tctcttgca gtcagcccaa  
ggctgcccc tccgtcactc tgttcccgcc ctctctgag gagcttcaag ccaacaaggc  
cacactgggtg tgtctcataa gtgacttcta cccgggagcc gtgacagtgg cttgaaagc  
agatagcagc cccgtcaagg cgggagtgga gaccaccaca ccctccaaac aaagcaacaa  
caagtacgcg gccagcagct atctgagcct gacgcctgag cagtgggaagt cccacagaag  
ctacagctgc caggtcacgc atgaaggag caccgtggag aagacagtgg cccctacaga  
atgttcatag tagtcccact ggggatgcaa tgtgaggaca gtggttcctc accctccctg
```

**Figure 13. Modification of the chicken light chain locus using the ET system.**

A chicken genomic BAC clone with the full length light chain locus was modified by homologous recombination. In a first step  $C\lambda$  was deleted by insertion of a selection cassette which was in a second homologous recombination step exchanged against the human  $C\lambda$  gene. The homology stretch was 50bp.

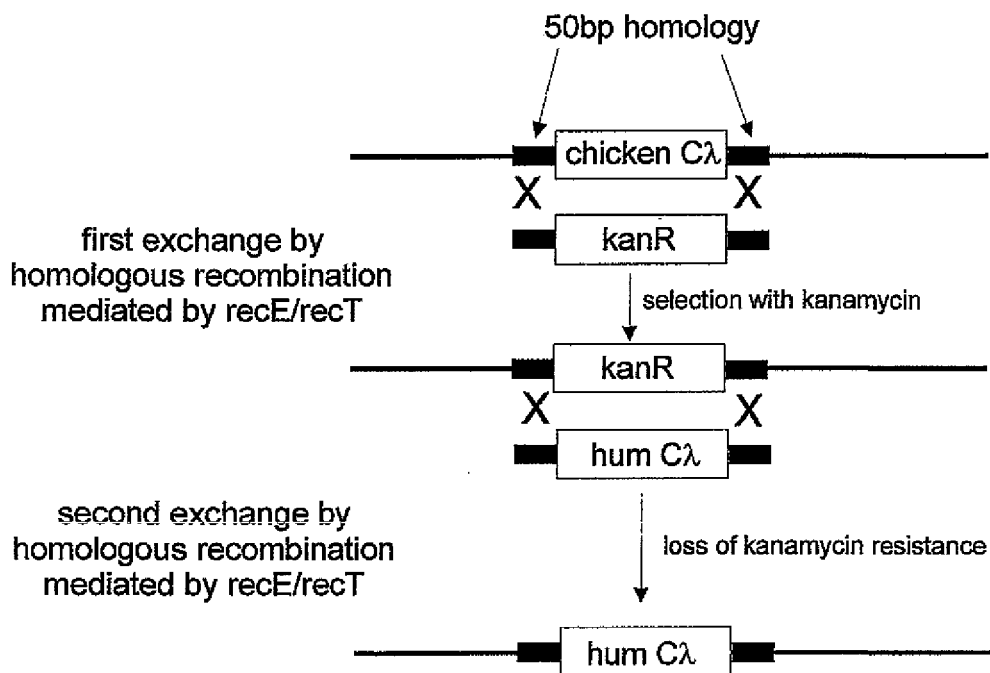


Figure 14. DNA fragment (SEQ ID NO: 58) containing a VJ gene segment with 80% sequence identity with chicken V gene segments and encoding a human VJ immunoglobulin polypeptide. Flanking sequences derived from chicken immunoglobulin DNA sequences are underlined.

.ttgccgttt tctccctct ctcctctccc tctccaggtt cctgggtgca gtcagtgtg actcagccgc  
cctcgggtgc agcagcccg ggacaagaag tcacgatctc ctgctccggg tctagtagca acattggcga  
taatttcgtc tcttggtac agcagctgcc tggcactgcc cctaagcttc tgatctatga taacaacAag  
agaccctcgg gcatccctga cggattctcc ggttccaaat cggcacctc agccacatta ggcatcactg  
ggctccaaac cggcgacgag gctgactatt actgtgggac tggggacagc agcctttctg ttggtatggt  
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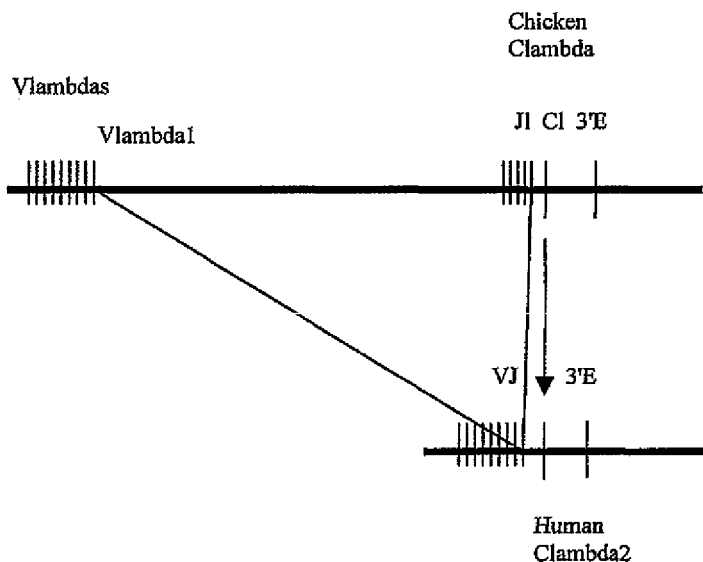


Figure 15. Humanized chicken light chain locus.